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THE INHIBITION OF OXIDATIVE AND PHOSPHORYLATIVE ENZYMES IN RAT LIVER MITOCHONDRIA BY AMINOAZOBENZENE DERIVATIVES[1]

W. C. McMURRAY[2]

Abstract

The liver carcinogen, dimethylaminoazobenzene, inhibited in vitro the oxidation of a variety of pyridine nucleotide linked substrates of rat liver mitochondria without affecting the process of oxidative phosphorylation. Cytochrome *c* oxidase activity was not inhibited by the carcinogen, nor was the succinoxidase activity, but the phosphorylation accompanying succinate oxidation was uncoupled. Similar effects were noted with other aminoazobenzene derivatives, but did not appear to be correlated with the ability of the compounds to evoke tumors.

The site of the respiratory inhibition by dimethylaminoazobenzene appears to be at the level between reduced pyridine nucleotide and cytochrome *c* in the respiratory chain. Mitochondrial dehydrogenase activity was not inhibited, while the oxidation of reduced diphosphopyridine nucleotide was markedly decreased. The reduction of the electron acceptor, ferricyanide, by pyridine nucleotide linked substrates was also strongly inhibited but the reduction of tetrazolium compounds was not affected. The latter observations suggest that dimethylaminoazobenzene produces a metabolic block between reduced flavin and cytochrome *c* in the mitochondrial electron transport system.

Introduction

Cancer cells have long been known to possess an impaired oxidative metabolism. From considerations of the low respiratory capacity and high glycolytic activity which he observed in tumors Warburg (1) concluded that the primary lesion leading to carcinogenesis was an irreversible injury to respiratory enzymes with a compensatory increase in glycolytic metabolism to provide an anaerobic energy source. Warburg's theory has been hotly debated (2–5) and continues to provide a stimulus for experimenters in the cancer field.

Considerable evidence has been obtained suggesting that ingestion of hepatocarcinogens leads to a diminution of oxidase enzymes in the livers of experimental animals prior to tumor formation (6–8). Moreover, Kielley (9) recently reported that liver carcinogens of the aminoazobenzene family inhibit in vitro the oxidation of glutamic acid by liver mitochondria from riboflavin-deficient rats.

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[1] Manuscript received August 10, 1959.
Contribution from the Department of Cancer Research, University of Saskatchewan, and the Saskatchewan Research Unit of the National Cancer Institute of Canada, Saskatoon, Saskatchewan.

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The present author has observed a similar inhibition of mitochondrial oxidations by aminoazobenzene compounds in normal liver mitochondria. In view of the drastic changes produced in liver mitochondria by riboflavin deficiency (10) it was decided to investigate this inhibition further in mitochondria from normal animals, with a view to elucidating the mechanism.

Experimental

Male Sprague–Dawley rats (200 to 300 g) maintained on a stock diet of pellets made from a formula by Prof. J. M. Bell of the Department of Animal Husbandry, University of Saskatchewan, were used throughout this study. Liver mitochondria were isolated according to the method of Schneider (11). The standard reaction mixture for oxidative phosphorylation studies contained 40–60 μmoles of substrate, 40 μmoles of potassium phosphate buffer (pH 7.4), 15 μmoles of $MgSO_4$, 5 μmoles of ATP,* 500 μmoles of sucrose, mitochondria from 0.5 g of rat liver, and 0.1 ml of 95% ethanol or aminoazobenzene compound in 0.1 ml of 95% ethanol in a total volume of 3.0 ml. The aminoazobenzene compounds were added last with a blow pipette. It was essential to obtain good dispersions in order to obtain consistent results. The reaction was started by the addition of 1 mg yeast hexokinase and 50 μmoles of glucose after a 10-minute preincubation. Oxidation and phosphorylation were determined for the subsequent 20-minute period at 30° C as described by Lardy and coworkers (12, 13), with the exception that inorganic phosphate was estimated on trichloroacetic acid extracts by the method of King (14). All conditions were determined on duplicate samples, and the results are expressed as the averages of duplicate determinations.

In the experiments using ferricyanide as electron acceptor the trichloroacetic acid extract was subjected to two extractions with two volumes of ethyl acetate which completely removed DAB, and the absorbance of the aqueous phase was determined at 420 mμ. Standard ferricyanide (and ferrocyanide) solutions prepared in this manner showed no change other than a slight dilution factor for which a correction was applied. In the presence of ferricyanide, inorganic phosphate was estimated by the method of Ernster *et al.* (15).

Phosphate-treated mitochondria were prepared by a modification of the method used by Hunter and Ford (16). In this procedure mitochondria from 0.25 g of liver were suspended in 1.0 ml of 0.02 *M* potassium phosphate (pH 7.4) – .05 *M* sucrose and incubated at 30° C for 10 minutes. After centrifugation of the resulting suspension at 8000×*g* for 10 minutes the mitochondrial pellet was resuspended in 0.25 *M* sucrose. This treatment resulted in more complete removal of pyridine nucleotides than the original method of Hunter and Ford (16).

Where DPNH was to be determined in extracts containing DAB as in the glutamic dehydrogenase and DPNH oxidase assays the reaction was stopped by the addition of one volume of acetone. After deproteinization at room temperature the DAB was extracted with two volumes of ethyl acetate, the phases were separated by centrifuging and the aqueous phase was filtered prior to determination of the absorbance at 340 mμ. DPNH standard solutions remained stable during this treatment.

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*The following abbreviations are used: ATP and AMP, adenosine tri- and mono-phosphate; DPN and DPNH, oxidized and reduced diphosphopyridine nucleotide; DAB, 4-dimethylaminoazobenzene; 2-Me-DAB and 3′-Me-DAB, 2-methyl- and 3′-methyl-4-dimethylaminoazobenzene; AB, aminoazobenzene; 3′-Me-AB, 3′-methylaminoazobenzene.

For the determination of the formazans resulting from reduction of tetrazolium compounds, the same deproteinization procedure as above was used and the absorbance was read directly at 540 mμ on the aqueous-acetone extracts after filtering. Since the absorption of DAB was low at this wavelength, the ethyl acetate extraction was not necessary and a small correction for the DAB absorption was applied instead. In some cases it was necessary to extract the precipitated protein with acetone to remove residual formazans.

Yeast hexokinase (Type II), yeast alcohol dehydrogenase, ATP, DPNH, cytochrome *c*, and most of the substrates employed were obtained from the Sigma Chemical Co., St. Louis, Mo.; DPN and AMP were products of Pabst Laboratories, Milwaukee, Wis.; neotetrazolium was obtained from General Biochemicals Inc., Chagrin Falls, Ohio. The recrystallized aminoazobenzene derivatives DAB, 2-Me-DAB, and 3′-Me-DAB were synthesized according to the general procedures of Miller *et al.* (17). Other aminoazobenzene compounds were prepared in Dr. J. A. Miller's laboratory.

Results

Oxidation of Glutamate and Succinate

The curves presented in Fig. 1A show the inhibition of mitochondrial glutamate oxidation obtained with various levels of aminoazobenzene com-

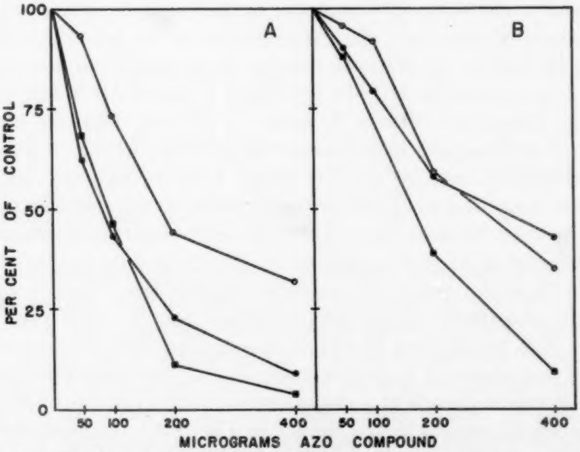

FIG. 1. The effect of varying concentrations of aminoazobenzene derivatives on (A) the oxygen consumption with glutamate as substrate, and (B) the P/O ratio with succinate as substrate. The values are expressed as percentage of the control values. Concentrations are expressed as μg/3 ml. ○——○ DAB, ●——● 3′-Me-DAB, ■——■ 2-Me-DAB.

pounds.* 3′-Me-DAB, which is a more potent carcinogen than DAB (18), produced greater inhibitions of glutamate oxidation. However, 2-Me-DAB, which is reportedly non-carcinogenic (18), also produced a strong inhibition.

Table I summarizes the effects of these and other aminoazobenzene compounds on mitochondrial oxidation. The relative carcinogenicity of the compounds studied is included for comparison (17, 18). The carcinogenic compounds DAB and 3′-Me-DAB, and the slightly carcinogenic 3′-Me-AB, as well as the non-carcinogenic 2-Me-DAB and AB all inhibited respiration with glutamate as substrate (Table I, expt. 1). With DAB the phosphate uptake was decreased to the same extent as the oxygen uptake so that no effect upon the P/O ratio was observed. In the presence of the other aminoazobenzene compounds phosphate uptake was too low to be measured accurately. However, lower concentrations of 2-Me-DAB and 3′-Me-DAB which only partially inhibited the respiration (Table I, expt. 2) did not decrease the P/O ratio. In a series of 21 separate experiments addition of 400 μg of DAB inhibited glutamate oxidation from 35 to 82% with a mean of 44% inhibition.

TABLE I

Effect of aminoazobenzene derivatives on glutamate oxidation and phosphorylation by liver mitochondria

Expt. No.	Azo compound added	Hepato-carcino-genicity	Oxygen uptake		Phosphate uptake, μmoles	P/O ratio
			μatoms	% inhibition		
1	None	–	8.89	—	24.4	2.75
	DAB (400 μg/3 ml)	+++	4.00	55	11.8	2.72
	2-Me-DAB (400 μg/3 ml)	0	0.39	96	—*	—
	3′-Me-DAB (400 μg/3 ml)	+++++	0.76	91	—*	—
	AB (400 μg/3 ml)	0	0.78	91	—*	—
	3′-Me-AB (400 μg/3 ml)	+	0	100	—*	—
2	None		12.1	—	31.1	2.57
	2-Me-DAB (50 μg/3 ml)		8.19	32	24.3	2.96
	3′-Me-DAB (50 μg/3 ml)		7.50	38	21.8	2.90
	2-Me-DAB (100 μg/3 ml)		5.58	54	14.4	2.62
	3′-Me-DAB (100 μg/3 ml)		5.27	56	17.4	3.30

NOTE: Incubated as described in Methods with 40 μmoles of L-glutamate as substrate.
*Differences were too small to be measured accurately.

A different situation was encountered when succinate was the substrate oxidized. As may be seen from Table II high concentrations of aminoazobenzene compounds, shown earlier to inhibit glutamate oxidation markedly, produced little or no effect upon respiration in the presence of succinate. However, the phosphate uptake was inhibited greatly, resulting in decreased P/O ratios. This uncoupling of the oxidative phosphorylation with succinate as substrate was observed with carcinogenic and non-carcinogenic derivatives. The effects of various levels of aminoazobenzene compounds on the P/O ratios in the presence of succinate are shown in Fig. 1B. Aminoazobenzene compounds in concentrations similar to those which inhibited glutamate oxidation

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*Since all the aminoazobenzene derivatives studied separated out during incubation it was not possible to express their molar concentrations in solution in the incubation medium. Accordingly the concentrations are expressed as micrograms of compound added.

TABLE II

Effect of aminoazobenzene derivatives on succinate oxidation and phosphorylation by liver mitochondria

Azo compound added (400 µg/3 ml)	Oxygen uptake, µatoms	Phosphate uptake, µmoles	P/O ratio	% inhibition of P/O
None	13.8	22.7	1.65	—
DAB	14.2	14.0	.99	40
2-Me-DAB	9.8	1.1	.11	93
3′-Me-DAB	12.2	8.9	.73	56
AB	13.1	9.9	.76	54
3′-Me-AB	12.9	1.2	.09	95

NOTE: Incubated as described in Methods with 60 µmoles of succinate as substrate.

decreased the P/O ratio during succinate oxidation. In a series of 20 experiments with succinate 400 µg of DAB decreased the P/O ratio from 27 to 65% with a mean decrease of 45%.

Other Substrates

The effects of DAB upon the oxidation of a variety of other substrates are shown in Table III. As was observed with glutamate 400 µg of DAB markedly inhibited the oxidation of the majority of these substrates without affecting greatly the P/O ratio. The oxidation of the Krebs cycle intermediates α-ketoglutarate, citrate, and isocitrate was strongly inhibited by DAB as were the DPN-linked oxidations of pyruvate and β-hydroxybutyrate to acetoacetate. Addition of DAB inhibited the oxidation of proline, which also requires DPN as coenzyme (19, 20), and of the fatty acid, caprylic acid. The oxidation of ascorbic acid in the presence of cytochrome *c*, which probably represents cytochrome oxidase activity (21, 22), was not affected by DAB, nor was the phosphorylation at this level of the electron transport system. This finding coupled with the negative effect upon succinoxidase activity made it appear

TABLE III

Effect of DAB on oxidation and phosphorylation by liver mitochondria with various substrates

Substrate	DAB (400 µg/3 ml)	Oxygen uptake		P/O ratio
		µatoms	% inhibition	
α-Ketoglutarate	−	7.86		2.79
(40 µmoles)	+	3.18	55	2.93
Citrate	−	5.36		2.88
(40 µmoles)	+	3.01	44	2.80
D,L-Isocitrate	−	9.19		2.96
(40 µmoles)	+	5.65	38	3.34
Pyruvate	−	6.55		3.74
(40 µmoles)	+	3.25	51	3.44
D,L-β-Hydroxybutyrate	−	6.31		2.96
(60 µmoles)	+	3.00	52	2.58
Proline	−	8.98		2.92
(30 µmoles)	+	4.71	48	2.67
Caprylate	−	4.39		0
(10 µmoles)	+	1.71	61	0
Ascorbate (30 µmoles)	−	4.65		0.97
+ cytochrome *c* (.3 µmoles)	+	4.85	0	0.98

unlikely that DAB interfered with cytochrome oxidase activity but rather inhibited some reaction involved in the oxidation of pyridine nucleotide linked substrates prior to cytochrome *c* in the respiratory chain.

Effect of DPN

Emmelot and Bos (23) had shown previously that the azo compound *o*-aminoazotoluene, a potent hepatocarcinogen in the mouse, inhibited glutamate oxidation by mouse liver mitochondria. This inhibition was overcome by the addition of DPN to the incubation medium. Moreover, Kielley (24) has shown that the liver carcinogen, acetylaminofluorene, acts as a competitive inhibitor for DPN in mitochondrial glutamic dehydrogenase. In view of the involvement of DPN-linked reactions in the respiratory inhibition by DAB in our experiments the effect of added DPN was tested, and shown to produce a similar reversal of the inhibition.

In order to explore this point and to test for a possible competition between DAB and DPN, mitochondria were depleted of their pyridine nucleotide content by the phosphate treatment described by Hunter and Ford (16). The effect of various concentrations of DPN upon glutamate oxidation and upon the inhibition produced by DAB is shown in Table IV, expt. 1. The inhibition of glutamate oxidation observed at low DPN levels was overcome as the concentration of DPN was increased. An extended series of DPN concentrations was then studied in order to carry out a Lineweaver–Burk analysis to test for true competition between DAB and DPN. In the course of these experiments a control flask containing DPN and the usual additions but no glutamate was studied and shown to yield high rates of respiration. Further experimentation showed that this high "endogenous" respiration was observed only in the presence of DPN, the yeast hexokinase used as high-energy phosphate-trapping system, and the ethanol normally added as a control for the DAB, which was added in ethanol (see Methods). It was further shown that the commercial yeast hexokinase contained alcohol dehydrogenase as a contaminant allowing oxidation of ethanol in the presence of DPN by the mitochondrial preparation (see also Maley (25)).

Table IV, expt. 2, shows the results of an experiment in which hexokinase was replaced by AMP as a phosphate acceptor. The rates of oxidation cannot be compared directly with those in expt. 1, since a different mitochondrial sample was employed; however, it may be seen that in the absence of hexokinase, the same degree of inhibition of glutamate oxidation was obtained with DAB in the presence of high or low concentrations of DPN. Experiment 3, Table IV, shows a direct comparison of the DAB inhibition with the same mitochondrial sample in presence of high DPN.

These findings indicated that the oxidation of ethanol was insensitive to DAB inhibition. A direct test of this possibility is demonstrated in Table V. Ethanol oxidation (which required the addition of DPN) was not inhibited by DAB in the presence of hexokinase or AMP. It may be concluded that the apparent competitive relation between DPN and DAB in glutamate oxidation

TABLE IV

Effect of DPN and DAB on glutamate oxidation by phosphate-treated mitochondria

Expt. No.	Phosphate acceptor system	DPN added (μmoles/3 ml)	DAB (400 μg/3 ml)	Oxygen uptake	
				μatoms	% inhibition
1	Hexokinase + glucose	0.2	−	5.78	
			+	3.23	44
		0.5	−	7.73	
			+	5.89	24
		2.0	−	10.0	
			+	8.98	10
2	AMP (20 μmoles)	0.2	−	5.20	
			+	3.12	40
		0.5	−	7.29	
			+	4.49	38
		2.0	−	10.5	
			+	6.29	40
3	Hexokinase + glucose	2.0	−	13.0	
			+	11.8	9
	AMP (20 μmoles)	2.0	−	9.80	
			+	5.60	43

TABLE V

Effect of DAB on ethanol oxidation by phosphate-treated mitochondria

Phosphate acceptor system	DAB (400 μg/3 ml)	Oxygen uptake, μatoms
Hexokinase + glucose	−	7.43
	+	8.21
AMP (20 μmoles)	−	8.34
	+	8.80

NOTE: Incubated as described in Methods with 20 μmoles of DPN, 1 mg of yeast alcohol dehydrogenase (100,000 units), and 0.1 ml of 95% ethanol as substrate.

was simply due to the DAB-insensitive oxidation of ethanol at high DPN concentrations.

Glutamic Dehydrogenase

Results of the experiments described up to this point have indicated that mitochondrial oxidations of a number of DPN-linked substrates are inhibited by DAB, at a site below cytochrome *c*. The possibility remained therefore that the inhibition occurred at the level of the mitochondrial dehydrogenases. Glutamic dehydrogenase was studied as representative of the pyridine-nucleotide dehydrogenases. A direct test of the effect of DAB upon the glutamic dehydrogenase activity of a mitochondrial extract proved negative under a variety of conditions.[*] It appears unlikely that DAB acts as a general inhibitor of DPN-linked dehydrogenases; if this were the case it should have inhibited glutamic dehydrogenase.

Ferricyanide and Tetrazoliums as Electron Acceptors

Ferricyanide appears to interact with the electron carrier system at the level of cytochrome *c* (26, 27) substituting for cytochrome oxidase as terminal

[*] Unpublished experiments.

electron acceptor. It may be seen from Table VI that with each of the DPN-linked substrates tested the reduction of ferricyanide was inhibited by DAB.

TABLE VI

Effect of DAB on ferricyanide reduction and phosphorylation by liver mitochondria with various substrates

Substrate	DAB (400 µg/3 ml)	Ferricyanide reduced		$P/2Fe(CN)_6^{-3}$ ratio	% inhibition of $P/2Fe(CN)_6^{-3}$
		µmoles	% inhibition		
Glutamate	−	33.6		.88	
(40 µmoles)	+	19.2	43	.82	7
α-Ketoglutarate	−	11.3		1.10	
(40 µmoles)	+	6.22	45	.96	13
Proline	−	16.5		1.39	
(30 µmoles)	+	9.26	44	1.49	0
Succinate	−	28.0		0.40	
(60 µmoles)	+	28.4	0	0.07	83

NOTE: Incubated as described in Methods with 50 µmoles of $K_3Fe(CN)_6$ and 50 µmoles of $KHCO_3$. Gassed with 95% N_2 − 5% CO_2.

The phosphorylation accompanying electron transfer in this segment of the respiratory chain was not impaired. By contrast ferricyanide reduction was not affected by DAB with succinate as substrate, while the phosphorylation was virtually abolished.

The results of an experiment using tetrazolium compounds as electron acceptors and with glutamate as substrate are shown in Table VII. These compounds probably accept electrons from the flavin component of DPNH − cytochrome *c* reductase (28, 29). On the basis of microequivalents of formazan produced per milliliter of enzyme per minute, neotetrazolium reduction was found to proceed at a rate about 10-fold greater than triphenyltetrazolium reduction. In neither case was tetrazolium reduction affected by the addition of DAB. Similar negative results were obtained when α-ketoglutarate was the substrate employed.

TABLE VII

Effect of DAB on tetrazolium reduction by phosphate-treated mitochondria with glutamate as substrate

Tetrazolium compound	DAB (400 µg/3 ml)	Formazan produced (µg)
Neotetrazolium	−	127*
(800 µg/3 ml)	+	123*
Triphenyltetrazolium	−	65†
(800 µg/3 ml)	+	62†

NOTE: Incubated as described in Table VI with tetrazolium compounds replacing ferricyanide, with 40 µmoles of glutamate as substrate, 2.0 µmoles of DPN and 20 µmoles of AMP replacing hexokinase + glucose.
*Incubated 5 minutes at 30° with 0.2 ml of mitochondria.
†Incubated 10 minutes at 30° with 0.5 ml of mitrochondria.

For technical reasons it was not possible to determine directly the effect of DAB upon cytochrome *c* reduction. Addition of the strongly absorbing dispersions of DAB made direct spectrophotometric determinations of reduced cytochrome *c* impossible, and solvent extractions of DAB after reaction destroyed the reduced cytochrome *c*.

DPNH Oxidase

The effect of DAB upon the enzymatic oxidation of DPNH was investigated next using a mitochondrial preparation that had been frozen and thawed, since such preparations exhibited a considerably higher DPNH oxidase activity than fresh mitochondria. Results of one such experiment are summarized in Table VIII. DAB inhibited the oxidation of DPNH to a similar extent (59%) as observed earlier with the oxidation of DPN-linked substrates. In two other experiments using different enzyme preparations DPNH oxidase was inhibited 54% and 63% by this level of DAB (400 μg/3 ml).

TABLE VIII
Effect of DAB on DPNH oxidation by frozen liver mitochondria

DAB (400 μg/3 ml)	DPNH oxidized/5 min	
	mμmoles	% inhibition
−	123	
+	50	59

NOTE: Incubated as described in Methods with 10 μmoles of AMP replacing hexokinase + glucose, with 0.2 μmoles of DPNH as substrate, and 0.05 ml of mitochondria at 28.5° C.

Miscellaneous

The inhibition produced by DAB on glutamate oxidation was not reversed by the following additions: flavin mononucleotide, flavin adenine dinucleotide or riboflavin, 10^{-4} *M*; menadione, 10^{-4} *M*; α-tocophenol acetate, 10^{-4} *M*; cytochrome *c*, 10^{-4} *M*; or uncoupling concentrations of dinitrophenol, 10^{-4} *M*.

Discussion

Results of the experiments reported in this paper are somewhat at variance with those described by Kielley (9) for a similar study but using riboflavin-deficient animals. Kielley (9) observed little or no inhibition by DAB of α-ketoglutarate or β-hydroxybutyrate oxidation in her preparations and concluded that DAB was a specific inhibitor of glutamate oxidation.

The findings reported here emphasize that the mitochondrial oxidation of all DPN-linked substrates is inhibited by DAB in vitro, although ethanol oxidation was an exception. It is possible that further oxidation of the product, acetaldehyde, which shows no requirement for DPN (25), is the DAB-insensitive step in ethanol oxidation. However, in unpublished experiments the author found that ethanol oxidation was not inhibited by DAB in the presence of excess semicarbazide sufficient to trap the acetaldehyde formed.

DAB was shown further to inhibit the mitochondrial oxidation of DPNH. The experiments with artificial electron acceptors indicate that DAB inhibits some component of the mitochondrial enzyme complex, DPNH − cytochrome *c* reductase. The observed effect of DAB on ferricyanide but not on tetrazolium reduction indicates that the block occurs at a point between flavin and cytochrome *c*.

The reduction of tetrazolium compounds by DPN-linked substrates probably represents a diaphorase-like activity (29, 30) rather than an estimate of DPNH – cytochrome *c* reductase activity. In this connection it is of interest that Yang *et al.* (31) noted that mitochondrial DPNH – cytochrome *c* reductase and tetrazolium reduction differed greatly in susceptibility to inhibition by chelating agents. The results described here are consistent with the hypothesis that DAB interferes with the single electron transfers of DPNH – cytochrome *c* reductase beyond the flavin level. These steps would be estimated with ferricyanide but not with tetrazolium as an electron acceptor.

The uncoupling effects produced by aminoazobenzene derivatives on the phosphorylation accompanying succinate oxidation are of some interest, particularly since the phosphorylations associated with DPNH oxidation were not affected. In certain cases the latter phosphorylations were also uncoupled by DAB, indicating a difference in degree of susceptibility of the two sites. For example, when treated with phosphate, a procedure that modifies membrane permeability (16), mitochondria were very sensitive to the uncoupling action of DAB regardless of the substrate employed (unpublished experiments). In addition DAB produced a 6- to 10-fold increase in the adenosine triphosphatase activity of fresh mitochondria (unpublished experiments), a property shared by the classical uncoupling agent, DPN (32). It appears likely that, in addition to its effect on respiratory enzymes, DAB is a true uncoupling agent, whose effectiveness depends upon the state of the mitochondrial membrane.

The experiments described with DAB have not all been repeated with other aminoazobenzene derivatives. There are sufficient lines of comparison, however, to make two general statements:

(1) The others tested among this family of compounds produced qualitatively the same effects as DAB.

(2) The potency of the compounds in producing respiratory inhibition and uncoupling of oxidative phosphorylation was not correlated with their ability to produce tumors.

It is always difficult to extrapolate from in vitro observations to the situation obtained in vivo. The inhibitions of mitochondrial respiratory and phosphorylative reactions observed in the in vitro experiments may be of no significance to the over-all carcinogenic process. Indeed, the concentrations required to produce the effects in our experiments in vitro would probably not be attained in the liver cell in vivo. For example, in unpublished experiments the author found that, 24 hours after feeding a rat 6 mg* of C^{14}-labelled DAB, only 0.3% of the dose equivalent to 18 micrograms of DAB was recovered from the whole liver. However, as shown by Kielley (9), glutamate oxidation by mitochondria from riboflavin-deficient animals is susceptible to lower concentrations of aminoazobenzene compounds, an observation compatible with the well-known observation that a high incidence of DAB-induced hepatomas depends upon a low level of riboflavin in the diet (33). This may be of some significance also with respect to the findings presented here suggesting that the respiratory block by DAB occurs at or near the level of flavin in DPNH – cytochrome *c* reductase.

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*This is roughly equivalent to the amount ingested daily in a diet which produces hepatomas in rats.

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References

1. Warburg, O. The metabolism of tumours. *Translated by* F. Dickens. Constable & Co., Ltd., London. 1930.
2. Potter, V. R. Advances in Enzymol. **4**, 201 (1944).
3. Greenstein, J. P. Biochemistry of cancer. 2nd ed. Academic Press, Inc., New York. 1954.
4. Weinhouse, S. Advances in Cancer Research, **3**, 269 (1955).
5. Warburg, O. Science, **123**, 309 (1956).
6. Potter, V. R., Price, J. M., Miller, E. C., and Miller, J. A. Cancer Research, **10**, 28 (1950).
7. Schneider, W. C., Hogeboom, G. H., Shelton, E., and Striebich, M. J. Cancer Research **13**, 285 (1953).
8. Allard, C. Proc. Can. Cancer Research Conf. **1**, 319 (1955).
9. Kielley, R. K. J. Natl. Cancer Inst. **19**, 1077 (1957).
10. Burch, H. B. and Hunter, F. E., Jr. Federation Proc. **18**, 199 (1959).
11. Schneider, W. C. J. Biol. Chem. **176**, 259 (1948).
12. Lardy, H. A. and Wellman, H. J. Biol. Chem. **195**, 215 (1952).
13. Copenhaver, J. H., Jr. and Lardy, H. A. J. Biol. Chem. **195**, 225 (1952).
14. King, E. J. Biochem. J. **26**, 292 (1932).
15. Ernster, L., Zetterström, R., and Lindberg, O. Acta Chem. Scand. **4**, 942 (1950).
16. Hunter, F. E., Jr. and Ford, L. J. Biol. Chem. **216**, 357 (1955).
17. Miller, J. A., Sapp, R. W., and Miller, E. C. Cancer Research, **9**, 652 (1949).
18. Miller, J. A. and Miller, E. C. J. Exptl. Med. **87**, 139 (1948).
19. McMurray, W. C., Maley, G. F., and Lardy, H. A. J. Biol. Chem. **230**, 219 (1958).
20. Lang, K. and Lang, H. Naturwiss. **18**, 493 (1957).
21. Maley, G. F. and Lardy, H. A. J. Biol. Chem. **210**, 903 (1954).
22. Lehninger, A. L., ul Hassan, M., and Sudduth, H. C. J. Biol. Chem. **210**, 911 (1954).
23. Emmelot, P. and Bos, C. J. Biochim. et Biophys. Acta, **24**, 442 (1957).
24. Kielley, R. K. J. Biol. Chem. **227**, 91 (1957).
25. Maley, G. F. J. Biol. Chem. **224**, 1029 (1957).
26. Pressman, B. C. Biochim. et Biophys. Acta, **17**, 273 (1955).
27. Devlin, T. M. and Lehninger, A. L. J. Biol. Chem. **219**, 507 (1956).
28. Brodie, A. F. and Gots, J. S. Science, **116**, 588 (1952).
29. Kun, E. Proc. Soc. Exptl. Biol. Med. **78**, 195 (1951).
30. Brodie, A. F. and Gots, J. S. Science, **114**, 40 (1951).
31. Yang, W. C., Yanasugondha, D., and Webb, J. L. J. Biol. Chem. **232**, 659 (1958).
32. Lardy, H. A. and Wellman, H. J. Biol. Chem. **201**, 357 (1953).
33. Kensler, C. J., Sugiura, K., Young, N. F., Halter, C. R., and Rhoads, C. P. Science, **93**, 308 (1941).

FACTORS INFLUENCING THE DISTRIBUTION OF PENTOSES IN THE ISOLATED RAT DIAPHRAGM[1]

N. Forbath and D. W. Clarke

Abstract

The addition of insulin, or of various enzyme inhibitors, to the intact isolated rat diaphragm causes an increase in pentose space, without causing a corresponding increase in inulin space or in total water. It is suggested that there is a relationship between cellular metabolism and permeability to pentoses. If pentose permeability and glucose permeability are governed by similar mechanisms, this means the glucose permeability might depend on cellular metabolism. However, phenethylbiguanide and pyruvate do not increase the permeability to pentoses, though they do increase glucose uptake. This means that glucose uptake might be increased without a corresponding increase in glucose permeability. Glucose utilization therefore may be governed by several factors of which the cellular permeability is only one.

Recent investigations, especially by Levine *et al.* (1), Park *et al.* (2), and Ross (3) have suggested that insulin acts by increasing the permeability of the cell to glucose. The mechanism of this action is still unknown, i.e., it is uncertain whether insulin primarily increases this permeability with other observed reactions following as a result of this, or whether insulin influences some reaction which might subsequently affect such permeability (4). One of the methods which have been used to study the action of insulin has been to measure the rate of entry into the cell of substances that are similar to glucose, but which are metabolized to a limited extent, if at all. Various workers have used certain pentoses in their studies, with the assumption that the mechanisms of entry of these pentoses and of glucose are similar. A good deal of evidence supports this concept (2, 5), though some experiments have indicated that there may be differences (5).

In the experiments to be described we have investigated some aspects of the relationship between cellular permeability to some pentoses and metabolism, using substances which, according to Randle (6), Haft and Mirsky (7), and Villee (8), increase the glucose uptake of the isolated rat diaphragm. These substances, unlike insulin, are potent enzyme poisons which either inhibit cellular respiration or uncouple oxidative phosphorylation.

Experimental

We used the intact diaphragm preparation of Kipnis and Cori (9) to measure permeability changes in the muscle. The insertion of the muscle to the rib cage, central tendon, and spine are intact, so tissue damage is minimized. The extracellular space is smaller than that found in the usual preparation of a rat diaphragm, and is closer to values found from in vivo measurements. In many other respects, however, the two preparations show at least a qualitative similarity in their reactions (9).

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Contribution from the Department of Physiology, University of Toronto, Toronto, Ontario. The results of the experiments outlined above were first presented at a meeting of the Canadian Physiological Society, June 9–11, 1958. During the preparation of the manuscript, the similar results of Randle and Smith (5) were published.

In most experiments, the diaphragms were incubated in a Dubnoff shaker for 1 hour at 37° C in 25 ml of Krebs–Henseleit bicarbonate medium, which contained 400 mg % pentose. The gas phase was 94% O_2 – 5% CO_2. If a phosphate buffer was used, the gas phase was pure O_2. The pH was between 7.2 and 7.4. After incubation the muscular portion of the diaphragm was dissected out and trimmed. A small, weighed portion was used for the dry weight determination. The greater part was extracted with boiling water for 10 minutes, and these extracts were used after deproteinization with zinc sulphate and barium hydroxide for the determination of pentoses, according to the method of Roe (10).

In preliminary experiments, we homogenized the muscle in ice-cold buffer and used this homogenate for the pentose determination. We later found that the extraction with boiling water gave identical results, and since this procedure is simpler and faster we have adopted it.

Inulin was added to the medium in a concentration of 200 mg % in the experiments in which its volume of distribution was determined. It was determined according to the method of Roe (11). In a few experiments the thiosulphate space was determined, but it was found that it was not a suitable index of extracellular volume changes, as the normal values were much too high.

Results

In Table I are summarized the effects of various substances upon the volume of distribution of some different pentoses. By volume of distribution we mean the volume of fluid necessary to contain that amount of pentose found in the tissue, at the same concentration as that in the medium.

The D-xylose space is significantly increased by insulin (1.5 units/ml), sodium arsenite (10^{-3} *M*), sodium cyanide (10^{-3} *M*), sodium azide (10^{-3} *M*), and dinitrophenol (2.5×10^{-4} *M*). Sodium fluoride (10^{-3} *M*) had no effect. Some of these enzyme poisons increase the glucose uptake of the cut diaphragm, according to Randle (6). They also increase the glucose uptake of the intact diaphragm preparation (5), though as Randle himself points out, one is actually measuring the glucose uptake of the diaphragm and all of the supporting structures which necessarily accompany this muscle in this particular preparation. Phenethylbiguanide, or DBI, a new hypoglycemic agent, in a concentration of 5×10^{-3} *M* does not significantly increase the xylose space, though at this concentration it definitely increases the glucose uptake of the cut diaphragm preparation (12, 13). For reasons just mentioned we prefer not to measure glucose uptake values in the intact diaphragm preparation. Similary, 0.01 *M* sodium pyruvate does not influence the penetration of D-xylose, though it does increase the glucose uptake (7).

Pentobarbital (5×10^{-3} *M*) increased the volume of distribution of L-arabinose, whereas phenethylbiguanide had only a very small effect.

According to the data of Levine (1), D-arabinose does not respond to insulin. In this preparation, however, we find that its volume of distribution is increased by insulin, by dinitrophenol, and by azide.

TABLE I

Pentose space in intact diaphragm (all values in ml/100 ml ± S.D.)

Additions to medium	Control	Cyanide	Arsenite	Dinitro-phenol	Azide	Fluoride	Insulin	Pyruvate	DBI	Pento-barbital
D-Xylose	53.7	74.6	78.2	69.5	66.0	49.1	74.3	57.0	53.4	
	±3.5	±5.9	±5.0	±4.9	±3.0	±6.6	±1.5	±5.1	±1.8	
p†		.001	.001	.001	.001	n.s.d.	.001	n.s.d.	n.s.d.	
N = No. of observations	15	7	6	7	7	4	4	4	5	
L-Arabinose	42.3								48.4	60.1
	±1.5								±4.3	±2.1
p†									.05	.005
N	4								4	4
D-Arabinose	40.8			52.4	49.1		66.0			
	±5.0			±2.1	±6.1		±8.1			
p†				.001	$p > .05$		.001			
N	4			5	4		4			
D-Xylose*	46.4			66.4	69.5		76.9		45.0	
	±2.6			±4.9	±1.6		±3.0		±7.8	
p†				.001	.001		.001		n.s.d.	
N	5			5	5		4		4	

*Phosphate buffer in incubation medium.
†Significance of difference between test value and control.
"n.s.d." stands for "no significant difference"

In all of the experiments just mentioned, bicarbonate buffer was used in the incubation medium. If the diaphragms are incubated in a Krebs–Ringer phosphate medium, the results for D-xylose are the same. However, Randle (14) and others (15, 16, 17, 18) have pointed out that in the cut diaphragm preparation, anoxia and dinitrophenol do not increase the glucose uptake when a phosphate medium is used.

Table II shows the effect of different enzyme poisons, pyruvate, and insulin upon the inulin spaces and upon the water content of the intact diaphragm. In general, these values are unchanged. In some cases certain inhibitors have caused statistically significant increases in inulin space or water content, but the magnitudes of these changes are too small to account for the measured differences in pentose space.

TABLE II

Inulin space and water content in intact diaphragm

Additions to medium	Control	Arsenite	Azide	Pento-barbital	Pyruvate	Dinitro-phenol	Insulin	DBI
Inulin space (ml/100 ml)	22.5 ±1.4	21.9 ±2.7	21.4 ±2.4	24.9 ±1.3	23.2 ±3.8	22.8 ±2.7	22.5 ±0.8	24.8 ±3.1
p*		n.s.d.	n.s.d.	.001	n.s.d.	n.s.d.	n.s.d.	n.s.d.
Water content (%)	76.5 ± .73	76.9 ± .62	77.8 ± .54	79.5 ± .55	76.3 ± .66	80.5 ± .56	75.5 ± .93	76.5 ± .65
p*		n.s.d.	.001	.001	n.s.d.	.001	n.s.d.	n.s.d.
N = No. of observations	8	8	8	8	4	8	4	8

*Figures show significance of difference between test value and control.
"n.s.d." stands for "no significant difference".

Discussion

These experiments show that various respiratory inhibitors or uncouplers of oxidative phosphorylation exert an influence upon the permeability of the isolated rat diaphragm to certain pentoses. The permeability change seems to be a fairly specific effect, because these poisons do not expand the inulin space. Similar permeability changes under the action of salicylate, another uncoupler, have been reported by Randle (14). These findings suggest that the metabolism of the cell and its permeability to pentoses may be related. With the assumption stated earlier in the paper, it is suggested that cellular metabolism and the permeability to substances, such as glucose, which can be utilized by the cell may be related. If the oxidative processes which make energy available to the cell are inhibited, then the cell is restricted to glycolytic processes for energy. An increased permeability to glucose might allow an increased anaerobic glycolysis, with a consequent supply of energy. The poisoning of the cell may create a situation similar to that found in exercise, where there is a relative oxygen lack. Helmreich and Cori (19) have shown that in such exercising muscles there is an increased permeability to pentoses.

The failure of phenethylbiguanide and pyruvate to increase the pentose permeability of the cell, coupled with their marked ability to stimulate glucose uptake (12, 13), suggests that glucose utilization of the cell may be governed by many factors, of which the permeability of the cell to glucose is only one.

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References

1. LEVINE, R., GOLDSTEIN, M. S., HUDDLESTON, B., and KLEIN, S. P. Am. J. Physiol. **163**, 70 (1950).
2. PARK, C. R., JOHNSON, L. H., WRIGHT, J. H., and BATSEL, H. Am. J. Physiol. **191**, 13 (1957).
3. ROSS, E. J. Medicine, **35**, 355 (1956).
4. HELMREICH, E. and CORI, C. F. Ciba Foundation colloquia on endocrinology. Vol. 9. J. & A. Churchill, Ltd., London. 1956. p. 227.
5. RANDLE, P. J. and SMITH, G. H. Biochem. J. **70**, 501 (1958).
6. RANDLE, P. J. and SMITH, G. H. Biochim. et Biophys. Acta, **25**, 442 (1957).
7. HAFT, D. E. and MIRSKY, I. A. J. Pharmacol. Exptl. Therap. **105**, 74 (1952).
8. VILLEE, C. A., WHITE, V., and HASTINGS, A. B. J. Biol. Chem. **195**, 287 (1952).
9. KIPNIS, D. M. and CORI, C. F. J. Biol. Chem. **224**, 681 (1957).
10. ROE, J. H. and RICE, E. W. J. Biol. Chem. **173**, 507 (1948).
11. ROE, J. H., EPSTEIN, J. A., and GOLDSTEIN, N. P. J. Biol. Chem. **178**, 839 (1949).
12. FORBATH, N. and CLARKE, D. W. Can. J. Biochem. and Physiol. **37**, 881 (1959).
13. WILLIAMS, R. H., TYBERGHEIN, J. M., HYDE, P. M., and NIELSEN, R. L. Metabolism, Clin. and Exptl. **6**, 311 (1951).
14. RANDLE, P. J. and SMITH, G. H. Biochem. J. **70**, 490 (1958).
15. PIERCE, F. T. and FIELD, J. J. Biol. Chem. **180**, 895 (1949).
16. VILLEE, C. A., DEANE, H. W., and HASTINGS, A. B. J. Cellular Comp. Physiol. **34**, 159 (1949).
17. WALAAS, E. and WALAAS, O. J. Biol. Chem. **195**, 367 (1952).
18. DEMIS, D. J. and ROTHSTEIN, A. Am. J. Physiol. **178**, 82 (1954).
19. HELMREICH, E. and CORI, C. F. J. Biol. Chem. **224**, 663 (1957).

THE EFFECT OF DIETARY MARINE FISH OILS ON THE SERUM CHOLESTEROL LEVELS IN HYPERCHOLESTEROLEMIC CHICKENS[1]

J. D. WOOD AND JACOB BIELY[2]

Abstract

An investigation was carried out into the effect of marine fish oils on the serum cholesterol levels in hypercholesterolemic chickens. The oils were fed at the 10% level in the diet. Lingcod liver oil and halibut liver oil prevented the hypercholesterolemic effect of supplementary cholesterol, whereas crude herring oil increased the hypercholesterolemia to the same extent as did corn oil and tallow. Ratfish liver oil, dogfish liver oil, and basking shark liver oil had less noticeable effects on the cholesterol levels. No clear explanation could be given for the observed behavior but there seemed to be a difference in the effects on serum cholesterol of liver oils from teleostei fish and from selachii fish, the former class of liver oils being much more potent in preventing the increase in serum cholesterol concentration in chickens caused by the addition of cholesterol to the diet.

Introduction

The effect of dietary constituents on cholesterol levels in the blood of both humans and animals has been studied extensively in recent years. Among the constituents studied in greatest detail are the fats and oils. It has been found that unsaturated vegetable oils in the diet lower the blood cholesterol levels in humans (1, 2, 3). Similar effects have been observed on the blood cholesterol level of rats made hypercholesterolemic by the addition of cholesterol and cholic acid to the diet (4). However, unsaturated vegetable oils have the opposite effect when they are fed to chickens. These species differences are reviewed by Stamler (5).

The exact nature of the compound or compounds present in the vegetable oil which is responsible for the changes in the cholesterol levels is not yet known. Some workers believe the effect is due to the fatty acid components of the triglycerides, more especially the polyunsaturated acids (6). On the other hand, Beveridge and co-workers (7) believe that the active factor is contained in the unsaponifiable fraction of the oil. Hegsted *et al.* (8) have postulated that the polyunsaturated fatty acids responsible for the decrease in serum cholesterol values must belong to the "essential" fatty acid class. These workers claimed that "non-essential" polyunsaturated fatty acids, such as eleostearic acid, had the opposite effect.

The fatty acid composition of marine fish oils is quite different from that of animal fats or vegetable oils. The marine oils contain moderately large amounts of highly unsaturated fatty acids (9) but little or none of the "essential" fatty acids. The amount of the highly unsaturated fatty acids varies considerably in oils from different species of selachii fish, the higher the unsaponifiable content of the oil, the less the degree of unsaturation of the component fatty

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acids (9). The unsaponifiable fraction of marine oils varies greatly from one type of oil to another, not only quantitatively but also qualitatively (10, 11, 12, 13).

The effect of individual fish oils on cholesterol levels has been reported. Anderson *et al.* (1) found that sardine oil depressed the serum cholesterol level in man, and de Groot and Reed (14) showed that cod liver oil had a similar effect in rats. They found that the unsaponifiable portion of the cod liver oil did not contain the depressant factor.

In view of the facts outlined above, an investigation was carried out into the effects of various marine oils on the serum cholesterol levels in chickens. The oils were selected so that a wide variation in the composition of the oils was obtained with respect to fatty acid composition, degree of unsaturation, and amount of unsaponifiable material. It was thought that such an investigation would yield useful information into the part played by the components of the oils in the alteration of serum cholesterol levels.

Materials and Methods

Birds

One-week-old New Hampshire cockerels which had been fed the standard diet were used for the experiments. The birds were standardized by weight to avoid any possibility of variations in serum cholesterol levels due to differences in the size of the chickens.

Diets

The standard diet was composed of wheat 54.9%, yellow corn 10%, oats 5%, wheat middlings 5%, wheat bran 5%, soybean oil (44% protein) 7.5%, meat meal (50% protein) 5%, herring meal (70% protein) 2%, distillers' dried solubles 2.5%, dehydrated cereal grass 1.25%, iodized salt 0.5%, limestone 1%, manganese sulphate 0.015%, feeding oil (2250A-300D/g) 0.25%, nicarbazine 0.05%. Riboflavin was added in the proportions 0.05 g/100 lb feed.

Cholesterol and oils were added to the standard diet as shown in Table II in the concentrations of 1% and 10% by weight respectively.

The fish oils employed in the investigation, with the exception of the herring oil, had been filtered through Hyflo Super-Cel to remove traces of water and other contaminants. The tallow used as a dietary supplement was Sta-Y-fat, which is a stabilized animal fat for use as a feed supplement (guaranteed 20 AOM minimum).

Experimental Procedure

The chickens were divided into groups of 20 and each group was placed in a separate cage and fed the appropriate diet and water ad libitum. After 7 days on the diets the chickens were bled from the wing and 0.5 ml blood collected from each bird. The blood from four birds was pooled to give a sufficient quantity for the cholesterol determinations and the serum collected in the normal manner. There were therefore five pooled samples of serum from birds on each of the diets. The procedure was repeated after 13 days on the experimental diets. The amount of each diet consumed by the chickens was measured after 7 days and 13 days.

Examination of Cholesterol in Blood Serum

The determinations were carried out using the method of Zlatkis, Zak, and Boyle (15). This method, not being specific for cholesterol, was shown to have limitations (16). Preliminary experiments were run and serum cholesterol determined by the above method, and by the more specific method of Sperry and Webb (17), both on the serum from birds fed the standard diet and on the serum from chickens eating the diets with cholesterol, tallow, and oil supplements. Similar results were obtained by both methods of analysis except that the Zlatkis, Zak, and Boyle method gave values which were 10% higher. The latter method was chosen for the routine analysis of the serum cholesterol, because it was less time consuming.

Analysis of the Fat and Oils

The iodine absorption number of the compounds was estimated using the Hanus method as described in the *Official methods of analysis* of the A.O.A.C. The amount of unsaponifiable material was determined as described in the same handbook.

Results

Analyses were carried out on the fat and oils which were used to supplement the diets of the chickens (Table I). The amount of unsaponifiable matter in the fat and oils was extremely variable. In corn oil, tallow, crude herring oil, and lingcod liver oil it amounted to a few per cent of the whole at the most, whereas in halibut, ratfish, dogfish, and basking shark liver oils there were large amounts of unsaponifiable material. The unsaponifiable fractions were solid except that from basking shark liver oil which was liquid at room temperature. The major unsaponifiable component of this oil is squalene (13), a C_{30} hydrocarbon containing six double bonds. Ratfish liver oil and dogfish oil differ from the other oils used in this investigation in that their high unsaponifiable content is composed mainly of the glyceryl ethers, chimyl, batyl, and selachyl alcohols (10, 12). The first of these compounds is saturated and the third has one double bond in its structure.

TABLE I

Composition of fat and oils used in the feeding experiments

Product	Iodine absorption number	Unsaponifiable material, %
Dogfish liver oil	114	14.3
Ratfish liver oil	90	26.1
Basking shark liver oil	203	42.9
Lingcod liver oil	143	3.7
Halibut liver oil	152	16.3
Crude herring oil	136	1.1
Corn oil	127	0.9
Tallow	63	0.5

The oils used to supplement the diets therefore varied considerably in composition. Some contained mainly triglycerides whereas others contained in addition large amounts of squalene or glyceryl ethers.

Table I also shows the iodine absorption numbers of the fat and oils. The oils chosen for this study exhibit a wide range of iodine values. The iodine absorption number in vegetable oils is often taken as a measure of the unsaturation of the component fatty acids because the oils consist mainly of triglycerides. The same does not apply to marine fish oils since the unsaponifiable material sometimes constitutes a significant portion of the oil and hence the degree of unsaturation of the unsaponifiable material strongly influences the iodine value of the oil. For instance, the high value observed here for basking shark liver oil is due to the high content of squalene (iodine value = 371). Similarly the low iodine values for dogfish and ratfish liver oils are due to their high content of the poorly unsaturated glyceryl ethers. The tallow, corn oil, and crude herring oil used in the investigation contained small amounts of unsaponifiable material and their iodine values give some indication of the amount of unsaturation in the component fatty acids. Tallow had the lowest degree of unsaturation followed by corn oil and herring oil in that order.

Table II gives the serum cholesterol levels of chickens which had been fed diets supplemented with cholesterol and various oils. The addition of 1% cholesterol to the standard diet produced hypercholesterolemia in the chickens which was evident after both 7 days and 13 days on the diet. These hypercholesterolemic chickens were used as the controls for the diets supplemented with the fat and oils because the latter diets also contained 1% cholesterol. The effects of the dietary supplements were the same after 7 days on the diets as they were after 13 days on the diets. Analysis of variance showed that after both time intervals the diets containing lingcod liver oil and halibut liver oil significantly lowered the serum cholesterol concentration below that of the controls. The addition of tallow, corn oil, and crude herring oil to the diets significantly increased the serum cholesterol levels. Ratfish liver oil, dogfish liver oil, and basking shark liver oil did not bring about changes which were comparable to those effected by the other oils. The diet supplemented with ratfish liver oil produced a decrease in the cholesterol concentration which was

TABLE II

The effect of dietary fat and oils on the serum cholesterol concentration in chickens

	Food consumed, g/chicken		Serum cholesterol, mg/100 ml	
Addition to the standard diet	7 days	13 days	7 days	13 days
None	97	193	185 ± 6	203 ± 27
1% cholesterol	95	191	314 ± 77	403 ± 51
1% cholesterol + 10% dogfish liver oil	68	156	252 ± 44	321 ± 119
1% cholesterol + 10% ratfish liver oil	75	179	217 ± 27*	262 ± 54*
1% cholesterol + 10% basking shark liver oil	79	190	313 ± 82	374 ± 96
1% cholesterol + 10% lingcod liver oil	57	117	192 ± 15**	155 ± 16**
1% cholesterol + 10% halibut liver oil	35	79	165 ± 12**	147 ± 21**
1% cholesterol + 10% crude herring oil	59	134	511 ± 65**	697 ± 161**
1% cholesterol + 10% corn oil	52	129	646 ± 140**	798 ± 219**
1% cholesterol + 10% tallow	52	136	432 ± 139**	662 ± 83**

*Analysis of variance indicates difference from "standard + 1% cholesterol" diet is significant at $P = .05$.
**Analysis of variance indicates difference from "standard + 1% cholesterol" diet is significant at $P = .01$.
NOTE: Each value in the table is the mean for five groups of serum ± the standard error.

significant at the 5% level of significance but not at the 1% level. The other two oils caused no significant change. Tallow and corn oil were included in the experiment for comparative purposes. Both these products increased the cholesterol concentration in serum to about the same extent as crude herring oil.

The amount of food consumed by the chickens is shown in Table II. The addition of fat or oils to the diet reduced the amount of food consumed because of the high energy content of the diet.

Discussion

The results presented here indicate that dietary marine oils vary greatly in their effect on serum cholesterol levels in chickens. No relationship appears to exist between the quantity of unsaponifiable matter in the oils and the serum cholesterol concentration. Dogfish liver oil and halibut liver oil contain approximately equal amounts of unsaponifiable matter but the former has no effect on cholesterol levels whereas the latter reduces significantly the cholesterol concentration. Basking shark liver oil has a very high unsaponifiable content but the oil has no effect on serum cholesterol levels. It may be argued that this is due to the comparatively small amount of triglyceride consumed by the chickens since the amount of that component was, at the most, 57% of the total weight of the oil. However, when the food consumption is taken into consideration it is seen that the amount of lingcod liver oil consumed was 62% that of the basking shark liver oil. In other words, the amount of triglycerides consumed by the chickens on these two diets was approximately the same, yet one oil had no effect and the other a marked effect on the serum cholesterol levels.

No relationship appears to exists between the iodine absorption number of the oils and their effect on serum cholesterol levels. For example, lingcod liver oil and crude herring oil have similar iodine values but they have opposite effects on the serum cholesterol. Tallow has a much lower iodine absorption number than either corn oil or crude herring oil but all three oils cause similar increases in the serum cholesterol concentration. However, the interpretation of the data is complicated by the fact that the unsaponifiable material influences the iodine value of the oil as mentioned previously.

There may be a possible difference between classes of fish in the effect of their oils on cholesterol levels. The results presented here indicate that the teleostei or bony fishes (lingcod, halibut) significantly lower the cholesterol content, whereas selachii or cartilaginous fishes (ratfish, dogfish, basking shark) have a much less pronounced effect. The degree of unsaturation of the component highly unsaturated fatty acids in liver oils of selachii decreases as the amount of unsaponifiable material increases (9), the oils with low unsaponifiable contents having a degree of unsaturation similar to that of teleost liver oils. This may explain the difference in effects of the teleostei and selachii liver oils observed here, because the latter oils were all high in unsaponifiable material and therefore low in the amount of unsaturation of their component fatty acids.

The addition of herring oil to the diet led to an interesting effect. Although herring is a teleost fish the addition of its oil to the diet of the chickens increased the serum cholesterol concentrations. However, this oil is not strictly comparable to the other teleost oils because, unlike the others, it was obtained from the whole fish rather than just the liver. In addition, the herring oil was crude and had not been subjected to the filtering operation which the other oils had undergone. A more thorough study of this topic will form the subject for a future investigation.

In summary it may be said that the results presented here indicate that there is no simple relationship between the amount or unsaturation of the component fatty acids in the oil and the serum cholesterol levels. However, the highly unsaturated fatty acids present in marine oils may play some part in reducing the cholesterol concentration. It would appear that "essential" fatty acids are not necessary for lowering cholesterol levels since fish oils contain little or none of this type of fatty acid. On the contrary, corn oil which contains large amounts of "essential" fatty acids produced the highest serum cholesterol levels observed during the investigation.

Acknowledgments

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References

1. Anderson, J. T., Keys, A., and Grande, F. J. Nutrition, **62**, 421 (1957).
2. Beveridge, J. M. R., Connell, W. F., and Mayer, G. A. Can. J. Biochem. and Physiol. **34**, 441 (1956).
3. Ahrens, E. H., Jr., Blankenhorn, D. H., and Tsaltas, T. T. Proc. Soc. Exptl. Biol. Med. **86**, 872 (1954).
4. Aftergood, L., Deuel, H. J., Jr., and Alfin-Slater, R. B. J. Nutrition, **62**, 129 (1957).
5. Stamler, J. Food Technol. **13**, 50 (1959).
6. Kinsell, L. W., Michaels, G. D., Friskey, R. W., and Splitter, S. Lancet, **1**, 334 (1958).
7. Beveridge, J. M. R., Connell, W. F., and Mayer, G. A. Can. J. Biochem. and Physiol. **35**, 257 (1957).
8. Hegsted, D. M., Gutsis, A., and Stare, F. J. J. Nutrition, **63**, 377 (1957).
9. Bailey, B. E. Marine oils with particular reference to those of Canada. University of Toronto Press, Toronto, Ontario. 1952. p. 27.
10. Morton, B. H. Fisheries Research Board Can. Progr. Repts. Pacific Coast Stas. **58**, 13 (1944).
11. Swain, L. A. Ph.D. Thesis, University of Washington, Seattle, Washington. 1945.
12. Swain, L. A. Fisheries Research Board Can. Progr. Repts. Pacific Coast Stas. **63**, 32 (1945).
13. Swain, L. A. and McKercher, B. H. Fisheries Research Board Can. Progr. Repts. Pacific Coast Stas. **65**, 67 (1945).
14. DeGroot, A. P. and Reed, S. A. Nature, **183**, 1191 (1959).
15. Zlatkis, A., Zak, B., and Boyle, A. J. J. Lab. Clin. Med. **41**, 486 (1953).
16. Rhodes, D. N. Biochem. J. **71**, 26P (1959).
17. Sperry, W. M. and Webb, M. J. Biol. Chem. **187**, 97 (1950).

THE SELECTIVE PROTEOLYTIC ENZYME INHIBITORY ACTION OF BENZETHONIUM CHLORIDE[1]

IVAN T. BECK, E. PINTER, R. D. MCKENNA, AND H. GRIFF

Abstract

Acute hemorrhagic pancreatitis in humans is thought to be perpetuated by the autolytic processes catalyzed by trypsin and lipase. This study is an integral part of our search for trypsin and lipase inhibitors to be used in the treatment of this disease.

Benzethonium chloride was found to inhibit tryptic activity in vitro. The proteolytic activity of rabbit's serum was inhibited, and the inhibition was most pronounced 6 to 12 hours after the subcutaneous injection of the compound. Fibrinolysin was also inhibited in vitro but benzethonium chloride had no inhibitory action on chymotrypsin, pepsin, or lipase.

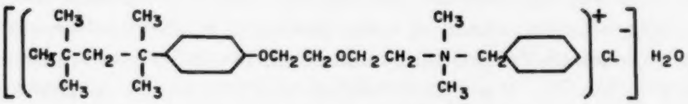
Serum proteins in vitro were precipitated only with very high concentrations of the compound. No significant protein changes were observed in sera of rabbits after the subcutaneous injection of the compound.

Introduction

The etiology of acute hemorrhagic pancreatitis or pancreatic necrosis is not known and is a controversial subject. It is agreed, however, that activation of trypsinogen to trypsin plays a role in the autolytic process (1). It is assumed that inhibition of tryptic activity will reduce the autolysis; therefore trypsin inhibitors may play a beneficial role in the treatment of the disease.

Previous experience with available trypsin inhibitors has not been encouraging (2, 3, 4). This may be due to the fact that these inhibitors themselves are of large molecular structure and may not reach the enzyme in the necrotic areas. Another reason for their ineffectiveness may be that they are slowly digested themselves and inactivated by trypsin (5).

For this reason, we started a search for chemicals of smaller molecular weight with possible trypsin inhibitory action. Calandra, Hardt, and Stanish (6) reported on a number of compounds with such activity, and benzethonium chloride has been mentioned among them. No details of the mechanism of its action have been reported as yet. The chemical structure of this compound is as follows.



Once the trypsin inhibitory action was established for this compound, it became desirable to elucidate its action on other proteolytic enzymes. Its action on lipase was also investigated to determine whether it might inhibit this esterase. If such had been the case one might have used the compound to inhibit both of the major enzymes involved in the autolytic process of acute pancreatitis.

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Contribution from the Gastrointestinal Research Unit, Royal Victoria Hospital, Montreal, Que.

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In order to investigate these aspects we tested the action of benzethonium chloride on trypsin activity in vitro, on the proteolytic enzyme activity of serum in vivo, and on chymotrypsin, pepsin, fibrinolysin, and lipase activity in vitro. The action of this compound on the proteins of serum and on crystalline trypsin was investigated using paper electrophoresis.

Materials

Trypsin, pepsin, chymotrypsin, lipase, fibrinogen, and thrombin were commercially available products and at the beginning of this study sufficient amounts of each were obtained to last through the entire experiment. Bovine plasmin activated with chloroform was obtained through the courtesy of Dr. Julian Ambrus and was used as fibrinolysin. Benzethonium chloride (benzyldimethyl 2-[2-(*p*-1,1,3,3-tetramethylbutylphenoxy)ethoxy] ethyl ammonium chloride) was obtained from Rohm and Haas, Philadelphia, under the trade name of "Hyamine 1622".

Methods, Experiments, and Results

(a) The Effect of Benzethonium Chloride on Trypsin Activity in vitro

Trypsin determinations were carried out using a modification of Anson's method (7). The amounts of hemoglobin originally suggested were found to yield unsatisfactory results and in our experiments a hemoglobin solution 1/10th the concentration suggested by Anson was used. The enzyme was dissolved in 1 ml of pH 7.5 phosphate buffer (anachemia) and incubated with 5 ml of hemoglobin solution. Results were expressed in units and one unit was considered to be the quantity of enzyme which liberates one microgram of tyrosine-like substance after 30 minutes' incubation at 37° C.

The inhibitory action of benzethonium in vitro was studied in the following manner. Before incubation, amounts of benzethonium chloride dissolved in 1 ml phosphate buffer (pH 7.5) were added to the enzyme and hemoglobin mixture so as to give final dilutions of benzethonium chloride of 1:1000; 1:5000; 1:10,000; 1:50,000; and 1:1,000,000. Two controls were run, one in which buffer solution instead of benzethonium was added before incubation and one in which the benzethonium and phosphate buffer solution were added only after incubation. It was soon observed that the two controls gave identical results. Therefore, controls in which benzethonium chloride was added after incubation were omitted from the experiments carried out later in the study. Results were expressed as percentage inhibition as compared to the appropriate control.

Inhibition of trypsin activity in vitro was found in all experiments. Figure 1 represents a typical experiment with different concentrations of enzyme and benzethonium chloride.

It can be seen that if the concentration of trypsin is high (1.5 mg per ml) the inhibition is less marked than with a lower concentration (0.5 mg per ml).

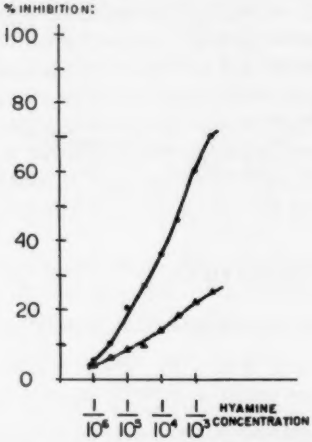

FIG. 1. Trypsin inhibitory action of benzethonium chloride in vitro.
Abscissa: Benzethonium chloride concentration (w/v).
Ordinate: % trypsin inhibition. Trypsin 0.5 mg/7 ml ● —— ●; trypsin 1.5 mg/7 ml △ .—.—△.

(b) The Action of Benzethonium on the Proteolytic Enzyme Activity of Serum in vivo

These studies were carried out in white rabbits of approximately 3000 grams body weight. Determinations were made as follows: samples of 2–3 ml of blood were obtained from each rabbit's ear on two occasions separated by 24 hours, prior to the administration of benzethonium chloride. The proteolytic enzyme activity was determined in each sample and expressed in trypsin units as described above. The average of these determinations was considered as 100% enzyme activity. After the subcutaneous injection of 15 mg/kg body weight benzethonium chloride dissolved in 0.9% saline, further blood samples were withdrawn so that some of the rabbits were bled at 1, 3, and 6 hours; others at 3, 6, and 9 hours; and others at 9, 18, 24, and 48 hours after the injection. In order to make a comparison between the different rabbits, the results obtained after benzethonium injections were expressed as percentage of the average pretreatment value of the same rabbit. Saline-injected rabbits were used as controls.

Figure 2 demonstrates that subcutaneous injection of 15 mg/kg of benzethonium chloride in a 2% solution causes an appreciable depression of the proteolytic activity of serum. This dose is far from the lethal dose of the drug, since we were able to inject 100 mg/kg body weight on repeated occasions into the same rabbit without any appreciable ill effect, except for a slight induration of the subcutaneous tissues. Statistical evaluation of the results of these experiments, using the p test, indicates that the enzyme level is significantly reduced during the first 6 hours following injection ($p = 0.02$) and the reduction is linear in time.

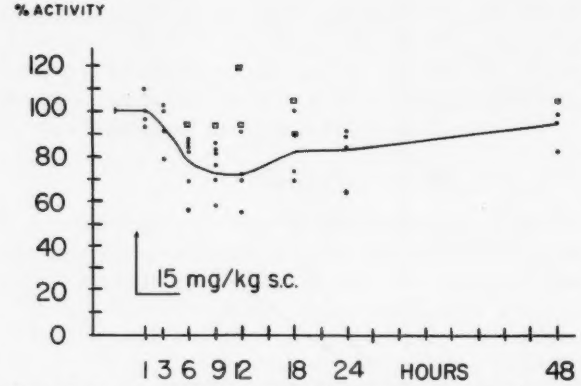

FIG. 2. Proteolytic enzyme inhibition of rabbit serum by benzethonium chloride in vivo.

Abscissa: hours after subcutaneous injection of 15 mg/kg benzethonium chloride.

Ordinate: % of preinjection proteolytic activity of serum.

Dots: values obtained after injection with benzethonium; the curve represents mathematical means of all these values.

Squares: values obtained after injection with 0.9% saline solution.

(c) The Effect of Benzethonium Chloride on Pepsin Activity in vitro

Pepsin determinations were made using the method of Anson and Mirsky (8) and inhibition was studied by a method similar to that described for trypsin in vitro. The pepsin concentration was 0.035 mg/ml and the pH was maintained at 1.5. It was found that the addition of benzethonium chloride in concentrations as high as 1:1000 caused no appreciable change in the pepsin activity.

(d) The Effect of Benzethonium Chloride on Chymotrypsin Activity in vitro

These studies were carried out using the same method as that described for trypsin inhibition in vitro with the exception that the pH was adjusted to 8.5, with the addition of sufficient amount of phosphate buffer pH 9.5. The additions of different concentrations of benzethonium to chymotrypsin did not appreciably change the activity of chymotrypsin.

(e) The Effect of Benzethonium Chloride on the Activity of Fibrinolysin in vitro

The method used for the study of fibrinolysin activity was based on that of Permin (9) with the following modifications: sterile fibrin clots were produced in Petri dishes by adding 200 units of bovine thrombin, dissolved in 2 ml of saline, to 8 ml of a pH phosphate buffer in which 60 mg of calcium chloride and 100 mg of powder containing 50% sodium citrate and 50% bovine fibrinogen were dissolved. A flat fibrin clot formed immediately. Chloroform-activated bovine plasmin was used as fibrinolysin. Plasmin (0.35 Loomis units (10)) was dissolved in 0.1 ml of saline and was dropped on a square of Whatman No. 3 filter paper measuring 10×10 mm. This square was placed in the middle of the fibrin plate. Results were expressed in square millimeters of digested area measured after 24 hours incubation at 37° C. Inhibition of this activity by benzethonium was tested by comparing the results obtained on the plates described above with those on fibrin plates prepared similarly, except that sufficient amounts of benzethonium chloride were added to the fibrinogen solution to give final dilutions of 1:1000 to 1:1,000,000 benzethonium. Flat fibrin clots could be prepared even after the addition of benzethonium in spite of the fact that clotting was considerably delayed by the higher concentrations of the compound. Since benzethonium chloride caused prolongation of the clotting time in these plates, it was concluded that the activity of the added thrombin is inhibited by the compound under the conditions of these experiments.

As to the action of benzethonium on fibrinolysin activity, Table I demonstrates that the fibrin-digesting capacity of chloroform-activated bovine plasmin is substantially reduced if benzethonium chloride is added to the fibrin plate.

TABLE I

Bovine plasmin (units)	Benzethonium final concn. (mg/cc)	Fibrin digestion no benzethonium added (in mm^2)	Fibrinolysin activity benzethonium added (in mm^2)	% fibrinolysin activity benzethonium added
0.035	$1:10^6$	596	94	15
0.035	$1:10^5$		94	15
0.035	$1:5\times10^4$		78	13
0.035	$1:5\times10^4$		78	13
0.070	$1:10^5$	3,516	176	5
0.070	$1:f\times10^4$		124	3

(f) The Effect of Benzethonium on the Activity of Lipase in vitro

Lipase determinations were carried out according to Goldstein *et al.* (11) except that the quantities used were reduced to 1/10th of those used in the original method. The action of benzethonium on lipase activity was tested by comparing the results of the control determinations with those obtained when benzethonium was added before the incubation. It was found that benzethonium does not influence the activity of lipase in vitro.

(g) The Effect of Benzethonium on Serum Proteins

During the course of these studies a chance observation was made. The addition of benzethonium chloride in concentrations of 1:100 to serum in vitro caused a precipitate to appear. We wished to find out whether proteins of benzethonium were precipitated. If benzethonium caused proteins to precipitate, its inhibitory action on certain enzymes may have been due to the denaturation and precipitation of these enzymes. This possible effect of benzethonium was investigated both in vitro and in vivo. In the in vitro experiments, benzethonium chloride was added to rabbit serum and the changes caused by the compound were evaluated electrophoretically. In the in vivo experiments, sera of rabbits injected with benzethonium chloride were studied.

Electrophoretic studies were carried out using a slight modification of the paper electrophoretic method described by Flynn and deMayo (12). Michaelis veronal buffer pH 8.5 was used. The potential gradient employed was 3 v/cm and the current was 0.4 ma/cm width. Running time was 20 hours at room temperature. The paper strips were stained with acid fuchsin solution and were evaluated by eluting the dye absorbed to the individual fractions. The amount of dye eluted was measured colorimetrically.

(1) In vitro Studies

Sera of rabbits were mixed with benzethonium chloride dissolved in 0.9% saline to obtain final concentrations of 1:100, 1:1000, or 1:10,000. A dense precipitate appeared immediately in tubes where the concentration was 1:100. Electrophoretic analysis of the supernatant fluid obtained by centrifugation showed that the concentrations of albumin decreased to values between 0 and 18% of their original levels. Total protein determinations showed a 12–29% decrease in these sera. This was considered as evidence that benzethonium chloride in a concentration of 1:100 is a precipitating agent affecting albumin alone while no changes in the globulin fractions were observed. When sera mixed with benzethonium chloride to obtain final concentrations of 1:1000 and 1:10,000 were tested in an identical manner, no change was observed either in the total protein values or in the distribution of the different fractions when compared with the electrophoretic pattern of these sera without the addition of benzethonium.

(2) In vivo Studies

Benzethonium chloride in a dosage of 15 mg/kg body weight was injected subcutaneously into eight rabbits. Their sera were subjected to electrophoresis twice before and at 6 and 9 hours after the injection. In both the 6- and 9-hour specimens the concentration of total proteins was similar to the pretreatment values. There was no significant change in the albumin values, but in three out of the eight rabbits between the β and γ globulin fractions a new fraction appeared. This was interpreted as splitting of the β globulin. There were no changes in the other protein fractions.

It is therefore concluded that at a dosage in which in vivo enzyme inhibition occurs, benzethonium chloride does not precipitate proteins and its activity is not due to a generalized protein denaturing action.

(h) The Effect of Benzethonium Chloride on the Electrophoretic Behavior of Crystalline Trypsin

This was tested as follows: 0.025 ml of 1% or 2.5% crystalline trypsin was mixed with an equal volume of benzethonium chloride in 0.9% saline. The final concentrations of benzethonium chloride were 1:100, 1:1000, 1:10,000, and 1:100,000. These mixtures were incubated for 10 minutes at 37° C to allow time for interaction of benzethonium and the enzyme prior to electrophoresis. The electrophoretic mobility of benzethonium alone was also determined.

When crystalline trypsin was mixed in vitro with benzethonium chloride to a concentration of 1:1000 or less, visible precipitation did not occur and the amount of protein in the solution did not vary from that when the same amount of trypsin was dissolved without the addition of benzethonium. (Total protein content of trypsin solution incubated with benzethonium was determined according to the method of Lowry (13).)

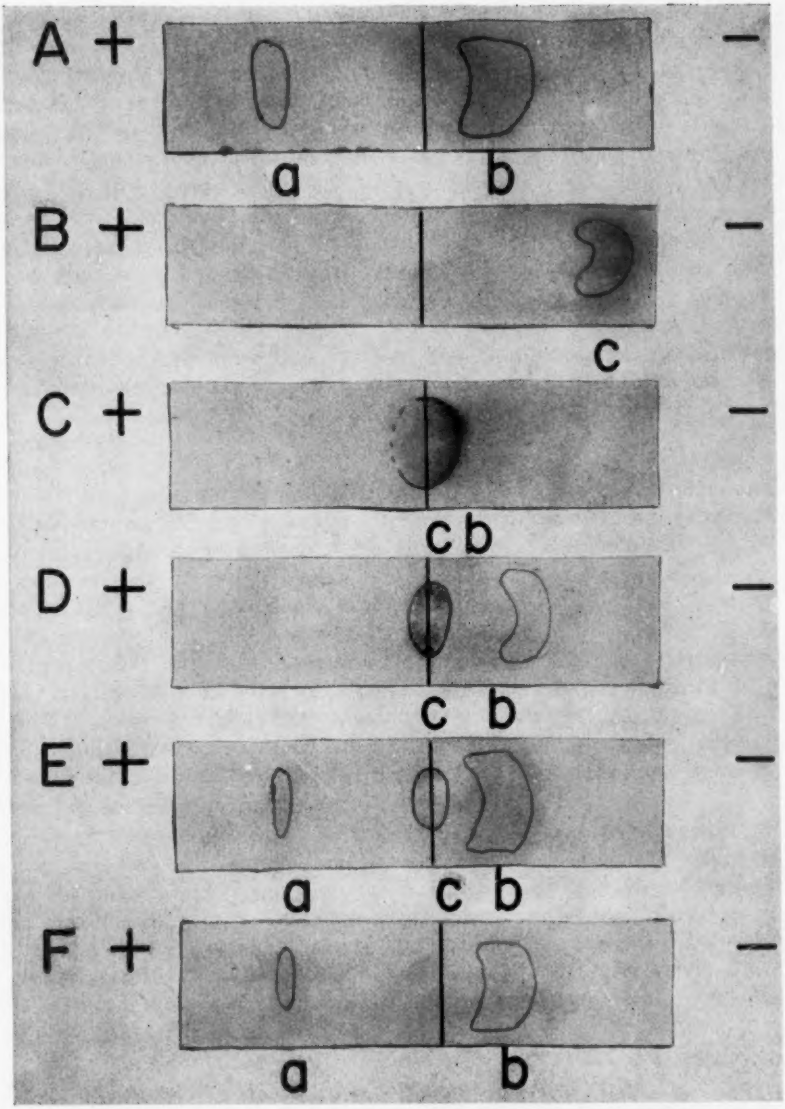

FIG. 3. Electrophoretic pattern of: (A) trypsin (1% soln.); (B) benzethonium chloride (1.5% soln.); (C) trypsin incubated with benzethonium chloride at a benzethonium chloride concentration of 1:100; (D) same as (C), concentration 1:1000; (E) same as (C) concentration 1:10,000; (F) same as (C), concentration 1:100,000.

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Benzethonium chloride itself is detectable with fuchsin staining and, when subjected to electrophoresis, the substance appears as a single fraction migrating to the cathode. When trypsin alone was subjected to electrophoresis, a separation of three fractions was obtained: a double fraction exhibiting a slight movement towards the cathode; a small, rather fast-moving band towards the anode; and, when greater concentrations of enzymes were used, a third fraction remaining at the starting line appeared on the strip. According to previous work carried out with the Tiselius apparatus, the fast-moving fraction corresponds to active trypsin while the others are autolytic products (14, 15, 16).

The effect of benzethonium on the electrophoretic pattern of the enzyme depends on the concentration of the drug. When 1:100 to 1:10,000 benzethonium dilutions were used, benzethonium remained at the starting line, the fast-moving enzyme fraction disappeared and the slow-moving fraction decreased in concentration. The absence of movement of benzethonium chloride indicated an interaction between the two substances. At lower benzethonium concentrations, there were no apparent changes in the enzyme pattern.

Discussion

Benzethonium chloride was shown to inhibit trypsin activity in vitro and proteolytic enzyme activity in the blood of rabbits injected with the compound. The questions which arise from these observations are: (*a*) What is the mechanism of these inhibitory actions? (*b*) Does the compound inhibit trypsin or some other proteolytic enzymes in the blood when injected into animals? As to the mechanism of action, we observed that the same amount of inhibitor will have a weaker action if the enzyme concentration is high. In other words, a higher concentration of inhibitor is necessary to produce comparable effects on a high concentration of trypsin than on a more dilute trypsin solution. This probably indicates a proportional molecular correlation between the enzyme and the inhibitor. Since the molecular weight of the trypsin used in our experiments is not known, it is impossible to calculate this correlation.

The observation that benzethonium in high concentrations precipitates proteins made us think that the inhibitory action of benzethonium might be due to its protein-denaturing action. Both chemical and electrophoretic studies have shown, however, that benzethonium chloride does not precipitate trypsin at concentrations which are effective in inhibiting proteolysis. Another fact which indicates that benzethonium chloride does not inhibit enzymes due to its protein-precipitating action is that some other proteolytic enzymes were not inhibited by the compound.

As to the specificity of the action of benzethonium chloride in the living animal, it was seen that, of the more common proteolytic enzymes in vitro, only trypsin, thrombin, and fibrinolysin were inhibited. It is felt, therefore, that lowered blood values after injection indicate inhibition of some but not all proteolytic enzymes.

Benzethonium chloride seems to be a promising agent for trial in acute pancreatitis and in states of increased fibrinolytic activity such as sometimes occur in hemorrhagic tendency in pregnancy or after chest surgery. The toxicity of benzethonium chloride, if administered subcutaneously, is low. Further work has to be done to try to diminish its local irritating action. The effect of benzethonium chloride when administered orally and its action on blood coagulation is under investigation.

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We are also indebted to Dr. G. I. Paul, Assistant Professor, Department of Genetics, McGill University, for the statistical evaluation of values presented in Fig. 2 and to Dr. J. S. L. Browne for helpful suggestions in the preparation of this manuscript.

References

1. Richman, A. Am. J. Med. **21**, 246 (1956).
2. Hoffman, H. L., Jacobs, J., and Freedlander, S. O. A.M.A. Arch. Surg. **66**, 617 (1953).
3. Rush, B., Jr. and Clifton, E. E. Surgery, **31**, 349 (1952).
4. Noskin, E. A., Popper, H. L., and Necheles, H. Rev. Gastroenterol. **19**, 731 (1952).
5. Laskowski, M. and Feng, C. W. J. Biol. Chem. **204**, 797 (1953).
6. Calandra, J. C., Hardt, L. L., and Stanish, E. S. Gastroenterology, **19**, 564 (1951).
7. Anson, M. L. J. Gen. Physiol. **22**, 79 (1938).
8. Anson, M. L. and Mirsky, A. E. J. Gen. Physiol. **17**, 393 (1934).
9. Permin, P. M. Acta Physiol. Scand. **20**, 388 (1950).
10. Loomis, E. C., George, C., Jr., and Ryder, A. Arch. Biochem. **12**, 1 (1947).
11. Goldstein, N. P., Epstein, J. H., and Roe, J. H. J. Lab. Clin. Med. **33**, 1047 (1948).
12. Flynn, F. V. and deMayo, P. Lancet, **261**, 235 (1951).
13. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. J. Biol. Chem. **193**, 265 (1951).
14. Liener, I. E. and Viswanatha, T. Biochem. Biophys. Acta, **22**, 299 (1956).
15. Timasheff, S. N., Sturtevant, J. M., and Bier, M. Arch. Biochem. Biophys. **63**, 243 (1956).
16. Bier, M. and Nord, F. F. Arch. Biochem. Biophys. **33**, 320 (1951).

STUDIES ON WHEAT PLANTS USING CARBON-14 COMPOUNDS

XI. THE RELATIVE INCORPORATION OF CARBONS 1, 2, AND 3 OF PYRUVATE[1]

W. B. McConnell, R. Nath,[2] and J. F. T. Spencer

Abstract

The incorporation of carbons 1, 2, and 3 of pyruvate into maturing plants has been compared by use of pyruvate-1-C^{14}, -2-C^{14}, and -3-C^{14} as tracers. The carbon-14 content of kernel proteins and lipids increased markedly when pyruvate was labelled in higher-number positions. The effect was strikingly demonstrated in the glutamic acid from the protein in which the specific activity varied in the proportion 1:5:10.5 when pyruvate labelled in the 1, 2, or 3 position respectively was administered. A small amount of administered pyruvate appeared to be utilized as the intact carbon skeleton for biosynthesis of starch and of alanine. It is considered, however, that most of the injected pyruvate undergoes decarboxylation, carbon-1 being utilized as carbon dioxide and carbons 2 and 3 as acetate.

Introduction

It was previously reported from this laboratory that labelling of wheat plants following injection of pyruvate-2-C^{14} was similar in many respects to that obtained after injection of acetate-1-C^{14} (1). The finding had followed upon the observation (2, 3), in the same system, that incorporation of the carboxyl group of acetate into the carbon skeleton of glutamic acid and related amino acids took place in a specific manner indicating utilization by way of the tricarboxylic acid cycle. It was reasonable, therefore, to consider the similarity of the results with pyruvate-2-C^{14} and acetate-1-C^{14} as evidence for dominance of the following general reaction:

$$CH_3—CO—COOH \xrightarrow{1/2\ O_2} CH_3—COOH + CO_2.$$

The present communication reports data from experiments designed for the further study of pyruvate utilization in the maturing wheat plants by observing the carbon-14 distribution following parallel injections of pyruvate-1-C^{14}, -2-C^{14}, and -3-C^{14}.

Experimental Methods

Thatcher wheat plants were seeded outdoors on May 18, and 79 days later they were injected with 0.1 ml of aqueous solution of carbon-14 labelled pyruvate (4). The injections were made into the hollow of the top internode on one tiller of each plant used. Ten tillers were injected with each of pyruvate-1-C^{14}, -2-C^{14}, and -3-C^{14}, the dose of carbon-14 being 9.1 μc per plant, and the weights of sodium pyruvate-1-C^{14}, -2-C^{14}, and -3-C^{14} injected were 0.215, 0.315, and 0.368 mg per plant respectively.

The labelled tillers were harvested 99 days after seeding (20 days after injection), at which time they had fully matured. The plants were air-dried,

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Contribution from the National Research Council of Canada, Prairie Regional Laboratory, Saskatoon, Saskatchewan. Presented in part at the Annual Conference of the Chemical Institute of Canada at Halifax, May 1959.
Issued as N.R.C. No. 5447.

[2] National Research Council of Canada Postdoctorate Fellow 1957–59.

separated into appropriate plant parts, and after a further drying period of 1 day in vacuum at 40° C they were ground to pass a 40-mesh screen.

The ground kernels were further separated into arbitrary fractions designated as starch, gluten, salt-soluble protein, ether-soluble material, and bran (or residue) according to the previously adopted scheme (4).

Certain amino acids were isolated (2, 5) from acid hydrolyzates of the gluten and each of these was decarboxylated with ninhydrin for separate recovery of carbon-1 (2, 6). The specific activities of the amino acids and of products obtained in their degradation were determined after combustion of the materials to carbon dioxide and counting the gas with the Dynacon vibrating reed electrometer (Nuclear-Chicago Corp.) using the 250-ml combustion chamber. The activity of other materials was obtained by plate counting at infinite thickness using an end-window counter (Micromil, Nuclear-Chicago Corp.). A factor for the conversion of the data of counting rate of plates to C^{14} content was obtained by comparison of plate counts with results obtained from combustion counting of representative samples of materials.

Methods used for degradation of amino acids have been previously described. Starch was hydrolyzed using strong acid, the glucose isolated by crystallization, and the carbon-14 distribution in its skeleton determined after degradation by fermentations with *Leuconostoc mesenteroides*, PRL 33 (7).

Experimental Results

Table I shows the over-all incorporation of carbon-14 into plant parts harvested as estimated by plate countings. Variations in C^{14} content among individual plants would likely be most evident in stems or leaf parts where mechanical effects such as slight leaks from injection wound and adsorption on tissue surfaces could occur. Comparison between the specific activities of these parts is thus not considered a reproducible measure of the biochemical utilization of tracer. Results suggest that carbon-3 is most effectively, and carbon-1 least efficiently, incorporated into the plant tissues. This is indicated, not only by the total carbon-14 content of the plants harvested, but also by the specific activity and total carbon-14 content of chaff and kernels which are well removed from the site of injection.

Compared to the previous experiment with pyruvate-2-C^{14}, the injections were made at a later stage (78 days after seeding compared with 71 days after seeding) and the interval between injection and harvest (20 days compared with 31 days) was shorter (1). This probably accounts, at least in part, for a lower carbon-14 content in the kernels and a larger proportion of recovered carbon-14 in other plant parts than was previously observed (4, 8). Some carbon-14, no doubt, remains in lower plant portions, but, since previous experience has indicated these to be of low specific activity (4) no attempts were made to analyze them.

Table II gives data on the specific activity of the wheat kernels and fractions isolated therefrom. The specific activity of the wheat was greater by a factor of about 1.8 when pyruvate-3-C^{14} was fed than when pyruvate-1-C^{14} was given. Labelling with pyruvate-2-C^{14} was intermediate. There appears to be little

TABLE I

Carbon-14 content of plant parts

Plant part	Weight, g/tiller	Counts* per minute	Total C^{14}, μc/tiller	% C^{14} injected
		Pyruvate-1-C^{14} (6 tillers)		
Stem†	.26	9,900	.20	2.2
Leaf and sheath‡	.17	1,100	.01	.1
Kernel	1.20	26,400	2.45	27.0
Chaff	.27	7,400	.15	1.6
Rachis	.06	12,000	.06	.7
			2.87	31.6
		Pyruvate-2-C^{14} (9 tillers)		
Stem†	.29	4,000	.98	10.8
Leaf and sheath‡	.20	5,400	.08	.9
Kernel	1.23	33,600	3.18	35.0
Chaff	.29	19,100	.43	4.7
Rachis	.07	21,400	.12	1.3
			4.79	52.7
		Pyruvate-3-C^{14} (10 tillers)		
Stem†	.24	38,200	.70	7.7
Leaf and sheath ‡	.17	19,500	.26	2.9
Kernel	1.08	46,500	3.86	42.4
Chaff	.25	24,800	.48	5.3
Rachis	.06	23,100	.11	1.2
			5.41	59.5

*Measured by plate counting ground samples of plant tissues with an end-window counter. Values are proportional to the specific activity of the samples.

†Stem portion from top node to base of head.

‡Upper leaf and sheath surrounding top internode.

difference among the tracers in their labelling of the starch and the bran. Differences in the specific activities of the kernels are thus accounted for in large part by notable differences in the labelling of the protein fractions, the specific activities of the gluten proteins isolated after pyruvate-1-C^{14}, -2-C^{14}, and -3-C^{14} feedings being in the proportion 1:3:5.5. The same order of preference for incorporation of the pyruvate carbons is observable in the salt-soluble proteins, and in the ether-soluble fraction.

The labelling of amino acids isolated from gluten hydrolyzates is shown in Table III, along with results on the labelling of their carboxyl groups. Glutamic acid shows the greatest differentiation between carbons 1, 2, and 3 of pyruvate, its specific activity from these three sources being in the proportion of 1:5.2:10.4. A slight preferential labelling of carbon-1 of glutamic acid by carbon-1 of pyruvate is indicated while the opposite effect is evident from carbon-3 of pyruvate. The labelling pattern in proline, as was shown in several previous experiments (1, 2, 3), is similar to that of the glutamic acid, from which it is presumed to be derived.

Aspartic acid is not extensively labelled but it appears that the terminal carbons are somewhat preferentially labelled by carbon-2 of pyruvate while carbons 2 and 3 are most radioactive when carbon-3 of pyruvate was labelled. The short-chain amino acids, glycine, alanine, and serine, are of intermediate specific activity and show less differentiation between the pyruvate carbons.

TABLE II

Recovery of carbon-14 from kernel fractions

Fraction	Weight recovered, g/g kernels	C^{14} in fraction, μc	Specific activity, mμc/mmole CO$_2$
	Pyruvate-1-C^{14}		
Kernels	1.000		54*
Starch	.590	1.22	59
Gluten	.102	.21	51
Salt-soluble protein	.045	.06	49
Ether-soluble material	.025	.05	37
Bran	.126	.15	32
		1.69	
	Pyruvate-2-C^{14}		
Kernels	1.000		74
Starch	.607	1.19	57
Gluten	.112	.72	154
Salt-soluble protein	.042	.10	90
Ether-soluble material	.020	.05	48
Bran	.113	.17	41
		2.23	
	Pyruvate-3-C^{14}		
Kernels	1.000		95*
Starch	.624	1.23	56
Gluten	.125	1.43	278
Salt-soluble protein	.051	.22	152
Ether-soluble material	.026	.14	90
Bran	.093	.15	42
		3.17	

*Estimated by plate counting.

TABLE III

Labelling of amino acids in gluten

	Specific activity, mμc/mmole CO$_2$					
	Pyruvate-1-C^{14}		Pyruvate-2-C^{14}		Pyruvate-3-C^{14}	
Amino acid	Total	C-1	Total	C-1	Total	C-1
Glutamic acid	52	79	270	280	540	230
Proline	65	95	214	270	500	260
Aspartic acid	56	46*	122	135*	260	153*
Threonine	19	15	67	64	160	
Glycine	121	148	144	129	142	140
Alanine	86	118	60	53	120	71
Serine	126	124	121	134	156	94
Valine	20		52	81	48	14
Tyrosine	11		91		90	
Phenylalanine	11		46		77	

*Average specific activity of carbons 1 and 4.

Valine and the aromatic amino acids are weakly labelled by carbon-1 of pyruvate but have incorporated significant amounts of carbons 2 and 3.

The formation of alanine from pyruvate has been demonstrated in many systems (10). Earlier work in this laboratory with acetate (2) failed to give results in accord with this idea. Furthermore, alanine isolated in the previous experiment with pyruvate-2-C^{14} was of relatively low specific activity and did not show the carbon-14 localization in carbon-2 to be expected from its formation by transamination from injected pyruvate-2-C^{14}.

Alanine isolated in the present experiments was also of comparatively low specific activity but the most radioactive carbon in each alanine sample corresponds to the carbon labelled in the pyruvate used as tracer (Fig. 1). The data indicate that some alanine is formed from the intact carbon skeleton of pyruvate injected. It is noted, however, that with each tracer less than 0.1% of injected carbon-14 can be accounted for in the alanine of gluten (gluten assumed to be 2.2% alanine (11)). It is estimated, using the excess radioactivity in the carbon of alanine corresponding to the labelled carbon of pyruvate as a measure, that only 0.015%, 0.014%, and 0.012% of labelled pyruvate-1-C^{14}, -2-C^{14}, and -3-C^{14} respectively have appeared in the alanine of gluten without rupture of the carbon chain. The average of the three values is about 0.014%. These approximations have considered only alanine in the major protein component of the kernel but, nevertheless, are adequate to show that only a very small proportion of the injected pyruvate carbon skeletons remains as alanine. It is noted that glutamic acid of gluten contained about 17 times as much of carbon-1 and more than 100 times as much of carbons 2 and 3 of pyruvate as did alanine.

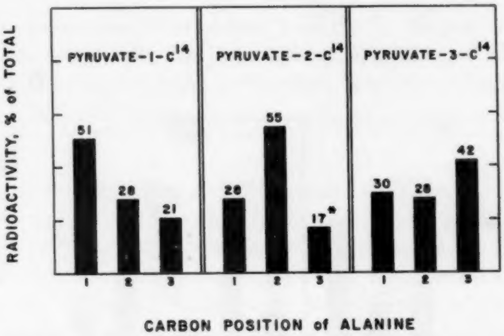

FIG. 1. Incorporation of pyruvate carbon into alanine of gluten. *Calculated by difference.

Table IV gives data on the distribution of carbon-14 in glucose obtained from the kernel starch. Although extensive "mixing" is evident, a tendency for some symmetry in the labelling of carbons 1, 2, and 3 with that of 6, 5, and 4 can be perceived. The similarity between the two "halves" of the glucose molecule was most evident when pyruvate-3-C^{14} was used as tracer. This is to be expected, in view of subsequent discussion, because this is the case when least interference from $C^{14}O_2$ would occur. To demonstrate the above relations more clearly the summation of radioactivity in carbons 1 and 6, 2 and 5, and

TABLE IV

Distribution of C^{14} in glucose obtained from kernel starch

Position of carbon	C^{14} content, % of total		
	Pyruvate-1-C^{14}	Pyruvate-2-C^{14}	Pyruvate-3-C^{14}
1	19	18	31
2	15	13	13
3	20	9	7
4	19	19	11
5	12	29	11
6	15	12	27

3 and 4 is shown (Fig. 2). Carbons 1 and 6 are labelled most effectively when pyruvate-3-C^{14} is fed; carbons 2 and 4 are best when pyruvate-2-C^{14} is given; and carbons 3 and 4 are most radioactive after administration of pyruvate-1-C^{14}. The results not only indicate that there is some direct incorporation of the three-carbon pyruvate skeleton into the glucose but also indicate that this occurs by condensation between carbons in position 1.

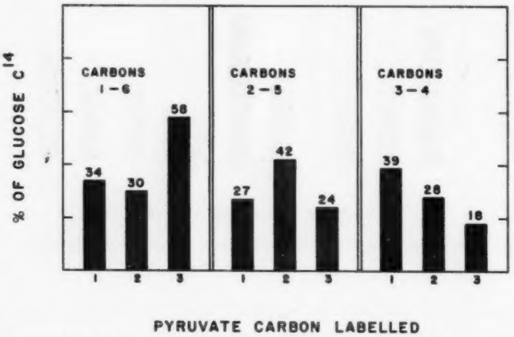

FIG. 2. Incorporation of pyruvate carbon into glucose of starch.

Table V gives data on the carbon-14 labelling of glutamic acid samples isolated in these and in previous experiments in which labelled pyruvate and acetate were given. Carbon-1 of glutamic acid contains more of the carboxyl carbon of pyruvate than do other carbons on the average (31% of glutamic acid carbon-14). This is to be expected if it is being utilized as $C^{14}O_2$ (12). Carbon-5 of glutamic acid contains 18% of the glutamic acid carbon-14 derived from carbon-1 of pyruvate leaving an average of about 16% for the remaining three carbons.

The two experiments with pyruvate-2-C^{14} and the one with acetate-1-C^{14} were done in different years and with somewhat different times of tracer injection and harvest (1, 2). The similarity among the results is nevertheless striking. Both tracers yielded glutamic acid of high specific activity compared with the wheat and with from 20 to 21% of the carbon-14 in carbon-1. A strong trend toward incorporation into carbon-5 of the glutamic acid is also noted. The similarity between the labelling with pyruvate-2-C^{14} and with acetate-1-C^{14} is considered in some detail in the earlier publication (1).

TABLE V

Labelling of glutamic acid from labelled wheat proteins

Tracer	Specific activity mμc/mmole CO$_2$*				% of C^{14} in glutamic acid	
	Wheat meal	Glutamic acid				
		Total	C-1	C-5	C-1	C-5
Pyruvate-1-C^{14}	59†	57	87	51	31	18
Pyruvate-2-C^{14}	81	297	308	685	20.7	46
Pyruvate-2-C^{14}‡		326	342	850	21	52
Acetate-1-C^{14}§	113	619	619	1950	20‖	63‖
Pyruvate-3-C^{14}	104†	593	247	130	8.5	4.4
Acetate-2-C^{14}§	144	839	342	200	8.2	4.8

*Calculated to values expected had tracer been given at the rate of 10 μc per plant.
†Based on plate counting.
‡Data for pyruvate-2-C^{14} taken from reference 1.
§Data taken from references 2 and 3.
‖Another series of analyses of C^{14} distribution in glutamic acid after feeding of acetate-1-C^{14} indicated a somewhat higher percentage in carbon-1 and lower percentage in carbon-5 (13). From 55 to 62% of the carbon-14 of glutamic acid was found in carbon-5.

The labelling of the glutamic acid by carbon-3 of pyruvate is now shown to be similar in several respects to that from carbon-2 of acetate. Not only is glutamic acid highly labelled as compared with other kernel components but the proportions of carbon-14 in positions 1 and 5 agree within the limits of experimental error. It appears furthermore that the relation between the specific activities of glutamic acid samples derived from radioactive carbons 3 and 2 of pyruvate is very like that of similar samples derived from radioactive carbons 2 and 1 of acetate.

Similar qualitative interrelations exist in the labelling of proline samples isolated but these have not been tabulated.

Discussion

All data presented are in accord with the general idea that decarboxylation is one of the major reactions undergone by pyruvate injected into the stems of maturing wheat plants. Arguments supporting the relation between the data and this conclusion require the simple and logical assumption that C^{14}O$_2$ is more readily lost from upper portions of the wheat plant than is acetate-C^{14} or other probable products of pyruvate metabolism. Since decarboxylation of pyruvate-1-C^{14} leads directly to C^{14}O$_2$ it follows then that this tracer should give the lowest over-all incorporation of carbon-14 (see Tables I and II). Carbon-1 of pyruvate was further observed to be more uniformly distributed among the various kernel constituents (Tables II and III). Since these were long-term experiments and since carbon dioxide is the primary carbon source for plants this lack of specific incorporation is also in harmony with the notion that C^{14}O$_2$ is a major product from pyruvate-1-C^{14}.

Such evidence as was noted for specific incorporation of carbon-1 of the pyruvate appears to be largely associated with utilization of the intact carbon skeleton of the pyruvate (slight excess labelling in carbons 3 and 4 of glucose and in carbon-1 of alanine (Table IV, Figs. 1 and 2)). It seems reasonable, however, to explain the presence of almost twice as much carbon-14 in carbon-1 of glutamic acid as in the average for other carbons after pyruvate-1-C^{14} injections by assuming $C^{14}O_2$ as an intermediate. Studies are currently being done in this laboratory on the distribution of carbon-14 in plants following exposure of either the leaves or the heads to carbon-dioxide-C^{14}. Results of this study may well assist in the interpretation.

The pattern of specific incorporation of either carbon-1 or carbon-2 of acetate into the glutamic acid carbon chain of protein was previously found to agree well with ideas of acetate utilization by way of the tricarboxylic acid cycle. It is significant for the present discussion that acetate-1-C^{14} not only was somewhat less specific in its labelling of kernel materials than was acetate-2-C^{14} but also yielded materials of lower specific activity . This seems to be satisfactorily explained by the fact that the carboxyl group of acetate is more readily converted to carbon dioxide than is the methyl group. The postulate that carbons 2 and 3 of injected pyruvate appear extensively as a two-carbon fragment equivalent metabolically to acetate thus not only satisfactorily explains the similarity between results with pyruvate-2-C^{14} and acetate-1-C^{14} and between results with pyruvate-3-C^{14} and acetate-2-C^{14} but also the intermediate position of pyruvate-2-C^{14} between pyruvate-1-C^{14} and -3-C^{14} both with respect to its specificity of labelling and its efficiency of incorporation.

The ratio of the specific activity of glutamic acid from pyruvate-2-C^{14} to that of glutamic acid from acetate-1-C^{14} was calculated using averaged data from earlier publications. The equivalent ratio for labelling of carbon-1 of glutamic acid was obtained and similar ratios taken for the labelling of proline and of carbon-1 of proline. The four fractions ranged from .58 to .65 and had a mean value of .61. A similar comparison between the effectiveness of pyruvate-3-C^{14} and acetate-2-C^{14} for labelling of glutamic acid and proline gave ratios of .64 and .75 respectively. A low value (.52) was obtained in comparing the labelling of carbon-5 of glutamic acid by pyruvate-2-C^{14} and acetate-1-C^{14}. This ratio, however, is depressed by including in the calculation the unusually high carbon-5 activity obtained in the first experiment with acetate-1-C^{14} (2). Had the calculation been based solely on the several results published later (13) the fraction would have been about .77. Considerable variation may occur in the labelling of glutamic acid under different conditions even with the same tracer, and hence, the above comparison must be regarded as only semi-quantitative. It appears that the injection of 1 μc of pyruvate-2-C^{14} or -3-C^{14} is equivalent as far as the labelling of glutamic acid or proline is concerned to the injection of from .60 to .75 μc of acetate-1-C^{14} or -2-C^{14} respectively. If, as seems reasonable, from data presented, the high labelling in the above amino acids after injection of labelled pyruvate occurs by way of a preliminary decarboxylation to acetate, it follows that about two-thirds of the injected pyruvate had undergone decarboxylation. Since higher ratios were found in comparisons involving more specific incorporation and less non-specific labelling by $C^{14}O_2$ (acetate-2-C^{14}) it is probable that the ratios found give a minimum value.

Acknowledgments

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References

1. Bilinski, E. and McConnell, W. B. Can. J. Biochem. and Physiol. **36**, 381 (1958).
2. Bilinski, E. and McConnell, W. B. Can. J. Biochem. and Physiol. **35**, 357 (1957).
3. Bilinski, E. and McConnell, W. B. Can. J. Biochem. and Physiol. **35**, 365 (1957).
4. McConnell, W. B. and Ramachandran, L. K. Can. J. Biochem. and Physiol. **34**, 180 (1956).
5. Hirs, C. H. W., Moore, S., and Stein, W. H. J. Am. Chem. Soc. **76**, 6063 (1954).
6. Van Slyke, D. D., MacFadyen, D. A., and Hamilton, P. J. Biol. Chem. **141**, 671 (1941).
7. Altermatt, H. A., Blackwood, A. C., and Neish, A. C. Can. J. Biochem. and Physiol. **33**, 622 (1955).
8. McConnell, W. B. and Bilinski, E. Can. J. Biochem. and Physiol. **37**, 549 (1959).
9. McConnell, W. B. Can. J. Biochem. and Physiol. **37**, 933 (1959).
10. Wang, C. H., Christensen, B. E., and Cheldelin, V. H. J. Biol. Chem. **213**, 365 (1955).
11. Pence, J. W., Mecham, D. K., Elder, A. H., Lewis, J. C., Snell, N. S., and Olcott, H.S. Cereal Chem. **27**, 335 (1950).
12. Tomlinson, N. J. Biol. Chem. **209**, 605 (1954).
13. Bilinski, E. and McConnell, W. B. Cereal Chem. **35**, 66 (1958).

STUDIES ON ALDOSTERONE

III. CHRONIC EFFECT ON THE BLOOD PRESSURE OF RATS[1]

A. G. Gornall, H. M. Grundy, and C. J. Koladich

Abstract

A rise in systolic blood pressure due to the administration of 0.4 or 0.5 μg of aldosterone per 100 g body weight to young male rats, over a period of 3 to 6 months, has been confirmed in two separate experiments. This effect was observed whether the aldosterone was given 3 days a week or 6 days a week, and whether dissolved in aqueous ethanol or in oil. Equal doses of 9-α-fluorohydrocortisone and of 2-methyl-9-α-fluorohydrocortisone produced similar though somewhat less consistent effects. When 4 or 5 μg of reserpine was administered along with aldosterone there was no clear evidence of a protective effect. Reserpine alone at these low dose levels was somewhat toxic in the rat and led to a rise in blood pressure.

The development of systolic hypertension as a cumulative effect of the administration of small doses of aldosterone over a period of months was first reported by Kumar *et al.* (1, 2). This potential pathogenic effect of the hormone was at first denied (3) but later affirmed by Gross *et al.* (4). They, however, used much larger doses and obtained the effect in a few weeks. The present report provides confirmation of the chronic effect of small doses of aldosterone on the blood pressure of rats and describes some related studies.

The data in this paper were obtained in two separate sets of experiments, the first extending from July 1956 to January 1957, and the second from February to August 1957. Both studies were carried out in intact male, albino rats (Carworth Farms Wistar strain) weighing initially about 100 g. They were kept two or three to a cage, in air-conditioned quarters at 75° F and fed ad libitum a Fox Cube* diet containing approximately 170 meq of sodium and 195 meq of potassium per kilogram. The drinking fluid was 0.5% saline.

The aldosterone used during most of these studies was the natural hormone isolated from aqueous commercial adrenal cortical extract according to the method of Gornall and Gwilliam (5). This fact made it necessary in most cases to limit the groups to five or six animals. For the 9-α-fluorohydrocortisone we are indebted to Dr. Josef Fried of The Squibb Institute, for the 2-methyl-9-α-fluorohydrocortisone to Dr. C. J. O'Donovan of the Upjohn Co., and for the reserpine (Serpasil), sesame oil, and later the DL-aldosterone acetate to Dr. C. W. Murphy of Ciba Co. Ltd.

Blood pressure measurements were made initially by the tail–cuff manometer method of Friedman and Freed (6) in the conscious resting animal. Without anesthesia this method was found to be very tedious and difficult, due to the interference of background noise picked up by the microphone attached to the tail. It is a little easier to detect the pulse sounds once the systolic pressure has become elevated. After many attempts to standardize

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Contribution from the Department of Pathological Chemistry, University of Toronto, Toronto 5, Ontario. This work was supported by grants from the Medical Division of the National Research Council, and from the Ontario Heart Foundation.

*Toronto Elevators Ltd.

this method for blood pressure measurement under light ether anesthesia we have accepted the conclusion of others that the results are too variable to demonstrate any except gross changes.

Because of these difficulties we turned to the photoelectric tensometer* method of Kersten *et al.* (7), which can be employed without anesthesia, and adopted it for routine work. This procedure also presents a number of technical problems. The instrument's circuit is a very sensitive one. It contains a matched set of tubes and photocell and such things as contacts and batteries need regular attention. When the photocell is covered with three thicknesses of paper the deflection produced by a clean microscope slide should be not less than five scale divisions. (Recently we have learned that it is advantageous to store the photocell housing in a desiccator when not in use. The instrument tends to be erratic in damp weather.)

Other difficulties are: (*a*) wrapping the cuff so as to obtain good occlusion of the vessels of the leg and (*b*) placing the rat's leg at an angle which minimizes pressing with the foot. These require experience and are a source of variation in results and of many unsuccessful attempts to obtain a blood pressure reading. We make it a rule never to take more than three readings with any single application of the cuff, and to accept an average pressure reading only when at least three or more trials have given similar results. One assistant, because of slight differences in technique, may obtain results that are consistently higher or lower than another. Control and test groups should therefore be followed by the same person. It was observed that some rats tolerate restraint in the holder more calmly than others. Whenever an animal becomes excited and refuses to remain quiet the pressure readings may rise 15–20 mm. Readings obtained under such conditions were rejected and the test repeated another day.

At the end of each experiment the rats were killed under ether anesthesia by exsanguination from the aorta. The heart, kidneys, and adrenals were dissected free of adherent fat, blotted dry, and weighed on a torsion balance. The tissues were then fixed, sectioned, and stained for histological examination.

Urine specimens were collected at intervals and tested for protein.

Results

Experiment I. Aldosterone Effect

Five intact male rats served as controls (group 3A). They received 0.1 ml of aqueous 15% ethanol subcutaneously 6 days a week. Five similar animals received 0.1 ml of 15% ethanol containing 0.4 μg of aldosterone per 100 g body weight 6 days a week (group 3B). The results are shown in Fig. 1 for the surviving four rats in the treated group and three control animals. During the first part of this study different methods of measuring blood pressure were being tested and attention is directed mainly to the values obtained in January 1957. The points plotted are averages of two or more satisfactory readings made on different days. On the left of the vertical line are readings made with the Friedman and Freed method without anesthetic (F). To the right of the

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*Metro Industries, Long Island City, New York.

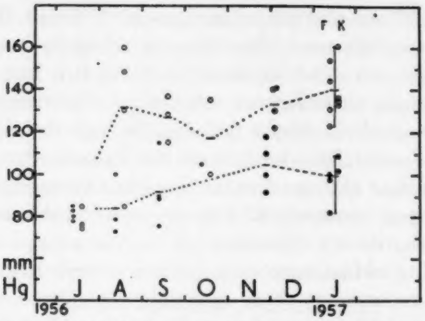

FIG. 1. Left ordinate: systolic blood pressure readings. Control rats, solid dots; aldosterone-injected rats, hollow circles. Abscissa: duration of experiment, July 1956 to January 1957. F, readings by Friedman and Freed method; K, by the procedure of Kersten *et al.*

line are the results by the method of Kersten *et al.* (K). It can be seen that the difference between the mean values is in either case about 40 mm.

Experiment II. Aldosterone Effect

The number of animals in experiment I was limited due to the fact that several small scale experiments were conducted concurrently. Where the results were significant the experiment was repeated and the results are reported here.

Following the adoption of the Kersten method for measuring blood pressure the effect of aldosterone was retested in larger groups of male rats. There were 15 control animals (group 4A); 9 received no injections, 3 received 0.1 ml of aqueous 15% ethanol, and 3 received 0.1 ml of sesame oil 6 days a week. Of the 18 aldosterone-injected rats 8 received 0.5 μg per 100 g body weight in aqueous 15% ethanol 6 days a week (group 4B(i)), 5 received the same dose on Mondays, Wednesdays, and Fridays only (group 4B(ii)), and 5 received their aldosterone 6 days a week in sesame oil (group 4B(iii)). The results are illustrated in Fig. 2. The readings in the injected and uninjected control animals could not

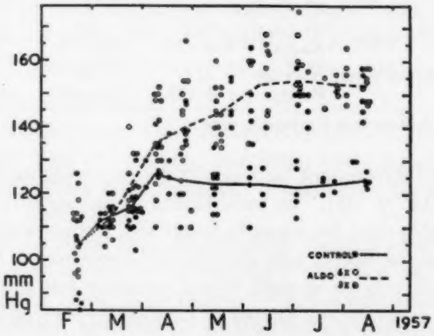

FIG. 2. Left ordinate: systolic blood pressure readings. Control rats, group 4A, solid dots; aldosterone-injected rats, hollow circles (groups 4B(i) and 4B(iii)) or circles with vertical bar (group 4B(ii)). Abscissa: duration of experiment, February to August 1957. Solid line, mean for the controls; broken line, mean for the aldosterone-injected animals.

be distinguished and are plotted as one group. Groups B(i) and B(iii) were plotted alike for the same reason. Readings for group B(ii) are shown separately although they too do not differ significantly from B(i) and B(iii).

It appeared to make no difference whether the hormone was administered in oil or in aqueous alcohol. About midway through the experiment synthetic aldosterone became available and in half the animals this material was substituted on a basis that 100 μg of DL-aldosterone-21-monoacetate (95% pure) was equivalent to approximately 42.5 μg of the natural hormone. The change did not produce a significant difference. What did appear surprising was that the animals receiving aldosterone only 3 days a week had just as marked an elevation of blood pressure as those receiving the hormone more often. The blood pressures of the aldosterone-injected rats were all elevated and averaged 30 mm higher than the controls.

Organ weights from the animals in these experiments are shown in Table I. In experiment I there does appear to have been some increase in heart and kidney weights; the differences between the mean weights for groups 3A and 3B are significant ($P < .05$). In experiment II no such differences were observed. It is probable that increases in heart and kidney weights are secondary and proportional to the degree and duration of elevation of the blood pressure. The effect of aldosterone on systolic blood pressure has not been sufficient to cause consistently a significant degree of cardiac hypertrophy.

TABLE I

Organ weights in control and aldosterone-injected male rats

Group (and number)		Body weight, g	Heart, g/100 g	Kidneys, g/100 g	Adrenals, mg/100 g
Controls (9)*	Average	477	.24	.50	7.3
3A	Range	346–564	.21–.29	.42–.60	5.1– 9.6
Aldosterone (4)	Average	409	.28	.58	8.5
3B	Range	376–462	.26–.29	.55–.61	6.8–10.2
Controls (9)	Average	446	.25	.56	7.9
4A(i)	Range	384–526	.23–.29	.52–.61	6.6– 9.6
Aldosterone (8)	Average	369	.25	.58	8.6
4B(i)	Range	353–418	.23–.29	.50–.62	6.6–10.0
Controls (3) (sesame oil)	Average	429	.25	.62	9.1
4A(iii)	Range	380–508	.24–.26	.61–.64	6.2–12.7
Aldosterone in sesame oil (5)	Average	455	.24	.53	8.2
4B(iii)	Range	388–523	.21–.27	.50–.55	6.8–11.3
Controls (3)	Average	518	.23	.62	7.5
4A(ii)	Range	464–624	.23–.24	.59–.63	6.9– 7.8
Aldosterone (5) (3 times per week)	Average	524	.25	.64	7.5
4B(ii)	Range	446–608	.21–.30	.60–.71	6.1–11.6

*This group includes six controls from previous experiment.

Experiment III. Effect of Fluorinated Hydrocortisones

Concurrently with experiment I, four rats (group 3N) were injected subcutaneously with 0.4 μg of 9-α-fluorohydrocortisone per 100 g body weight

6 days a week. Another four rats (group 3P) received 0.4 μg of 2-methyl-9-α-fluorohydrocortisone per 100 g body weight for the same 6-month period. In each case the steroid was dissolved in alcohol so that when the solvent was diluted to 15% concentration with water or saline the required dose was contained in 0.1 ml. The control rats of Fig. 1 served also as controls for these groups. The mean differences between control and treated rats are shown in Fig. 3. The data suggest that these synthetic steroids had a weaker and more transient pressor effect when compared to aldosterone, even though they are similar in salt-retaining potency. This finding can be compared with that obtained with desoxycorticosterone in our earlier study (2).

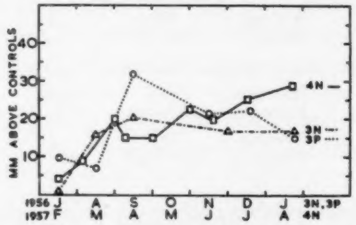

FIG. 3. Left ordinate: millimeters mercury by which the average systolic pressure of steroid-injected rats exceeded the mean for their respective controls. Abscissa: duration of the experiment, July 1956 to January 1957, for groups 3N and 3P, February to August 1957 for group 4N. Rats in groups 3N and 4N received 9-α-fluorohydrocortisone; group 3P, 2-methyl-9-α-fluorohydrocortisone.

The effect of 9-α-fluorohydrocortisone on blood pressure was reassessed in seven male rats (group 4N) concurrently with experiment II. The dose this time was 0.5 μg per 100 g body weight in 0.1 ml 15% ethanol given subcutaneously 6 days a week. The results of this study are presented also in Fig. 3. It can be seen that the pressor effect of 9-α-fluorohydrocortisone was roughly equivalent to that obtained in a parallel experiment with the same amounts of aldosterone (Fig. 2).

Organ weights of the animals in groups 3N, 3P, and 4N are shown in Table II. They can be compared with the control values from Table I. The increased average heart weights of group 3N, and kidneys of groups 3N, 3P, and 4N, are significant at the 5% level when compared with the controls.

Experiment IV. Effect of Reserpine

Concurrently with experiment I five rats received subcutaneously, along with 0.4 μg of aldosterone, 5 μg of reserpine per 100 g body weight 6 days a week. Ampoules of Serpasil containing 5 mg per 2 ml were diluted with water to contain the required dose in 0.1 ml.

The animals began to show toxic effects after about 3 months; two died and two were sacrificed after 4 months because they were not well. In this study it appeared that reserpine may have blocked the pressor effect of aldosterone, though in these animals, relative to uninjected controls, a tendency to an elevated blood pressure was apparent in the first few weeks. It was impossible to evaluate the extent to which both the blood pressure and the organ weights may have been affected by the impaired health of the animals.

TABLE II

Organ weights

Group (and number)		Body weight, g	Heart, g/100 g	Kidneys, g/100 g	Adrenals, mg/100 g
(A) Fluorinated hydrocortisone experiments					
Controls (18)	Average	443	.245	.53	7.9
from Table I	Range	346–564	.21–.29	.42–.61	5.1–10.2
3N (4)	Average	417	.29	.58	7.75
9-α-fluorohydrocortisone	Range	382–453	.27–.33	.55–.64	6.7– 8.6
3P (4)	Average	424	.26	.60	7.45
2-methyl-9-α-fluorohydrocortisone	Range	412–438	.24–.27	.56–.62	6.9– 7.9
4N(7)	Average	409	.25	.58	7.75
9-α-fluorohydrocortisone	Range	386–481	.21–.28	.56–.60	6.6– 9.3
(B) Reserpine and aldosterone experiment					
4R (6)	Average	381	.25	.57	9.7
Reserpine controls	Range	333–424	.23–.27	.55–.61	8.0–12.5
4RA (6)	Average	394	.24	.55	8.0
Reserpine + aldosterone	Range	345–428	.23–.26	.51–.57	6.8– 9.1

The study was repeated concurrently with experiment II, this time using a slightly lower dose of reserpine, 4 μg per 100 g body weight. A control series of six male rats (group 4R) received reserpine alone. The test series of six male rats (group 4RA) received in addition 0.5 μg of aldosterone per 100 g body weight. The blood pressure readings during this study are shown in Fig. 4.

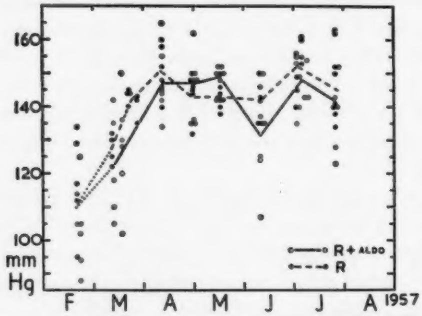

FIG. 4. Left ordinate: systolic blood pressure readings. Hollow circles, rats injected with reserpine (R) and aldosterone; solid dots, rats receiving reserpine alone. The solid and broken lines are means for the groups. Abscissa: duration of the experiment, February to July 1957.

It was surprising to find that at this dose level the chronic administration of reserpine caused an elevation of systolic pressure which was not enhanced by aldosterone administration. A somewhat similar effect of reserpine has been reported in dogs by Maxwell *et al.* (8). Even a dose of 4 μg of reserpine per 100 g body weight was found to be slightly toxic in the rat after several weeks, and when diarrhoea and weight loss occurred the dose was reduced temporarily to 2 μg. The mechanism of this pressor effect of reserpine seems worthy of further study.

The organs from the rats in this experiment were weighed as before and the results are given in Table II. There was no difference between the two groups.

The tissues from the rats in these experiments are being examined by Dr. W. G. B. Casselman and the findings may be published in detail elsewhere. The kidneys of the aldosterone-treated rats in group 3B could not be distinguished microscopically from those of the controls. But three of these four animals showed early proliferative changes in the coronary vessels. The same change was seen in three of the four rats in group 3N that received 9-α-fluorohydrocortisone. The kidneys of the rats in group 3N and group 3P showed slight-to-moderate proliferation of the epithelium of the renal tubules along the inner surface of Bowman's capsule. The zona glomerulosa of the adrenals of animals in groups 3B, 3N, and 3P was totally devoid of lipid.

Urine was obtained from the rats in experiments I, II, III, and IV and examined for protein. Abnormal proteinuria was found in one control rat (group 3A) and in three rats in group 4N. Two control rats receiving sesame oil showed proteinuria as did three of the rats in group 4B(iii) that received aldosterone in sesame oil.

Discussion

The cumulative effect of the chronic administration of small doses of aldosterone, confirmed twice in the experiments described above, is not readily explained. Although histological studies have yet to be completed, nothing thus far points to a primary effect on the kidney. Our impression at present is that renal changes, when they occur, are secondary to the elevation of blood pressure.

If it is true that slight, recurrent excesses of aldosterone can lead to a moderate elevation of systolic pressure, then the possibility of this hormone being an etiological or predisposing factor in human essential hypertension must be given serious consideration. It is easy to imagine that familial, psychic, environmental, metabolic, or endocrinological factors could give rise to chronic, or intermittent, overproduction or underdestruction of aldosterone. A degree of compensation for, or adjustment to, these excesses might allow a tendency to hypertension to persist for a long time in a benign form.

The effect produced by 9-α-fluorohydrocortisone, at the same low dose level as aldosterone, provides additional evidence for the hypertensive potentialities of small but chronic excesses of mineralocorticoids when associated with a relatively high intake of salt. Swingle *et al.* (10) found that 9-α-fluorohydrocortisone could produce hypertension in adrenalectomized dogs and Owen *et al.* (11) and others, have described a pressor effect in humans.

Our results with reserpine appear to be at variance with those reported by Gaunt *et al.* (9). At a dose of 10 μg/100 g body weight they observed an effect which was beneficial when compared with the more drastic action of large doses of desoxycorticosterone and salt. Their limited data with a 5 μg dose of reserpine are not incompatible with our own. It seems unlikely that the 10 μg dose would have been well tolerated by our animals.

References

1. Kumar, D., Hall, A. E. D., Nakashima, R., and Gornall, A. G. (Abstract) Rev. can. biol. **14**, 265 (1955).
2. Kumar, D., Hall, A. E. D., Nakashima, R., and Gornall, A. G. Can. J. Biochem. and Physiol. **35**, 113 (1957).
3. Gross, F., Loustalot, P., and Meier, R. Experientia, **11**, 67 (1955).
4. Gross, F., Loustalot, P., and Meier, R. Acta Endocrinol. **26**, 417 (1957).
5. Gornall, A. G. and Gwilliam, C. Can. J. Biochem. and Physiol. **35**, 71 (1957).
6. Friedman, M. and Freed, S. C. Proc. Soc. Exptl. Biol. Med. **70**, 670 (1949).
7. Kersten, H., Brosene, W. G., Ablondi, F., and SubbaRow, Y. J. Lab. Clin. Med. **32**, 1090 (1947).
8. Maxwell, R. A., Ross, S. D., Plummer, A. J., and Sigg, E. B. J. Pharmacol. Exptl. Therap. **119**, 69 (1957).
9. Gaunt, R., Antonchak, N., Miller, G. J., and Renzi, A. A. Am. J. Physiol. **182**, 63 (1955).
10. Swingle, W. W., Baker, C., Eisler, M., Le Brie, S. J., and Brannick, L. Endocrinology, **57**, 220 (1955).
11. Owen, J. A., Engel, F. L., and Wester, T. B. J. Clin. Endocrinol. and Metabolism, **17**, 272 (1957).

THE EFFECT OF INGESTION OF DISODIUM ETHYLENEDIAMINETETRAACETATE ON THE ABSORPTION AND METABOLISM OF RADIOACTIVE IRON BY THE RAT[1]

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Abstract

Disodium ethylenediaminetetraacetate (EDTA) was fed to young adult rats at a level of 0.1% of the diet. The effect on absorption, metabolism, and excretion of dietary iron was studied after administration of radioactive ferric chloride by stomach tube. The presence of EDTA decreased the absorption of iron and increased the excretion in urine. When EDTA was not fed until after the Fe^{59} had been absorbed, the iron appeared to be increased in the blood and the spleen, and to be removed from the liver.

Introduction

Ethylenediaminetetraacetic acid (EDTA) forms soluble non-ionized complexes with many metal ions (1). Salts of this compound will clarify wine (2) and will prevent after-cooking darkening in potatoes (3, 4), apparently by forming such complexes with iron. It may thus find important uses in certain food industries. A deterrent is the lack of knowledge concerning possible harmful effects from its periodic ingestion in small amounts over a long period of time.

It has been found that when salts of EDTA are ingested by men and rats 4 to 18% is absorbed (5, 6, 7). After parenteral injection it is practically all excreted in the urine (5, 6), and it has been demonstrated that under such conditions it increases the urinary excretion of calcium (8) and of iron (9).

Sodium salts of EDTA have been given to rats in single oral doses as high as 4 g per kg, and also fed at a level of 0.5% of their diets for 1 year without any ill effects (2). Human subjects have been given as much as 4 g intravenously to determine its effect on calcium metabolism with no overt toxic symptoms (8).

It appears that any deleterious effects from the continued use of EDTA in foods should be sought in its effect upon metals (6). Of primary importance in this respect would be their increased excretion in the feces, and consequently the possibility of deficiencies. It seems most important to consider the case of iron, since EDTA preferentially combines with iron in the pH range prevailing in that part of the alimentary tract from which it is absorbed (10). Some experiments have already been performed with Fe^{59} and "Fe-3 Specific" on rats (11) but in most of these the iron was administered parenterally, so that the effect of the sequestering agent on absorption was not studied.

The purpose of our experiments was to obtain information on the absorption and distribution of dietary iron when EDTA was ingested.

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Contribution from the Atlantic Regional Laboratory, National Research Council, Halifax, Nova Scotia.
Issued as N.R.C. No. 5439.

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Procedure

Rats with an average body weight of about 150 g were used. They were kept in special individual metabolism cages made from a large pyrex beaker with a double bottom of copper screen. This allowed the separate collection of urine and feces, but did not obviate radioactive contamination of urine by the feces. However, the consistency of the data for diverse trends in the urine and feces indicates that such contamination did not occur to a significant extent.

The percentage composition of the diet was as follows: casein 16, sucrose 69, corn oil 7, cellulose 5, $CaCO_3$ 0.85, NaCl 0.425, KCl 0.5, KH_2PO_4 1.25, $CuSO_4.5H_2O$ 0.0016, and $FeCl_3.6H_2O$ 0.0048.

Distilled water was supplied ad libitum.

The disodium dihydrate of EDTA was added to the diet of experimental animals at a level of 0.1 g per 100 g. As the iron content of the diet was about 0.001%, the ratio of the EDTA compound to iron was 100, which corresponds to a molar ratio of 15 and an equivalent ratio of 10.

Each rat was given 10 to 15 g of food per day, so that its intake of iron was 0.1 to 0.15 mg per day. Although this is a lower level of iron than the rat usually obtains, it is sufficient, and was used to produce more favorable conditions for studying its absorption and metabolism. The daily intake of EDTA in experimental animals was 10 to 15 mg.

The determination of radioactivity was done as follows: The heart, spleen, liver, and sternum were excised, washed in cold water, and dried at 110° on aluminum dishes. They were then counted whole with a Geiger–Müller tube at sufficient distance so that variations in the counting geometry due to variation in sample size and shape were small. The feces for each 24-hour period were similarly dried and counted. The daily collection of urine was diluted to 25 ml, filtered, and 100-μl aliquots were plated on aluminum planchets, dried,

TABLE I

The absorption and retention of Fe^{59} given to rats ingesting EDTA

Regimen	Rat No.	Relative counts per minute								
		Feces		Urine		Heart	Liver	Spleen	Sternum	Whole blood
		1st day	2nd day	1st day	2nd day	2nd day				
Experiment I										
No EDTA in diet	A1	3760	4420	*a*	2	123	1430	713	98	528
	A2	844	4160		19	120	2840	963	65	503
	A3	664	5460		6	120	1820	762	94	482
	A4	2990	2420		2	80	2160	1030	97	579
EDTA in diet	B2	13300	8570	*a*	47	16	280	36	13	50
	B3	9950	8420		129	14	137	89	9	40
Experiment II										
No EDTA in diet	A1	458	1440	60	18	58	730	661	81	*a*
	A2	615	521	77	16	75	649	1210	70	
	A3	355	1260	*a*	16	51	379	*a*	128	
	A4	226	1140	170	7	122	995	1600	115	
EDTA in diet	B1	1270	*b*	571	72	12	130	214	18	*a*
	B2	482	1500	340	46	4	99	*a*	9	
	B3	1260	695	305	45	26	248	124	65	
	B4	1280	*b*	608	48	19	273	97	32	
P values (experiment II)		0.02		0.02	<0.01	<0.01	<0.01		<0.01	

NOTE: *a*, not determined; *b*, no sample.

TABLE II

The effect of the addition of EDTA to the diet upon the distribution and metabolism of Fe^{59} in rats

		Relative counts per minute														
		Feces						Urine								
											Heart	Liver	Spleen	Sternum	Whole blood	Blood plasma
Regimen	Rat No.	1st day	2nd day	3rd day	4th day	5th day	6th and 7th day	4th day	5th day	6th and 7th day	7th day					
Experiment III																
No EDTA in diet	A1	869	2050	673	332	340	193	2	5	3	125	3220	759	101	655	1.7
	A2	3860	1110	606	266	132	78	5	2	2	114	2610	643	57	*a*	*a*
	A3	4170	3650	810	474	380	345	5	5	2	50	2010	950	78	408	1.0
EDTA added to diet after Day 4	B1	1340	4110	844	340	317	322	2	9	22	93	1630	1530	112	787	5.0
	B2	1650	3780	1440	327	267	137	2	5	14	90	1590	1450	104	618	3.4
	B3	2950	4800	*a*	355	190	120	1	8	14	138	1630	1450	84	737	4.2

Note: *a*, no sample.

and counted in a gas flow counter. Aliquots of blood were plated and counted in the same way. It should be stressed that the activities given are relative counts per minute, not absolute, so that while all the figures in an experiment for any type of sample may be compared, the different types of samples cannot be compared with each other. All activities were determined to a probable error of 2% or less.

In experiments I and II the animals were kept in the special cages for a preliminary period of 4 days. During this time they were fed the special diet, with or without EDTA. Each rat was then given by stomach tube 1.0 ml of a solution of $Fe^{59}Cl_3$. In experiment I this volume contained about 0.2 μg Fe with a total activity of 4.8 μc, and in experiment II about 0.4 μg Fe with a total activity of 11.0 μc. In experiment I the radio iron was supplied soon after the food for the day had been eaten; in experiment II it was supplied soon after one-quarter of the day's food allowance had been eaten. Feces and urine were collected each day for 2 days. The animals were then sacrificed, blood from the heart was removed and heparinized, and the liver, heart, spleen, and sternum were removed. The activities of all samples were determined and are recorded in Table I.

In experiment III the animals were kept for a preliminary period of 4 days in the special cages, but they were all fed the diet without EDTA. Each was then given by stomach tube 0.5 ml of a solution of $Fe^{59}Cl_3$ which provided about 0.3 μg of Fe^{59} with a total activity of 7.7 μc. The rats were starved for at least 6 hours before and after the administration of the radio iron so that the maximum amount would be absorbed. The rats were kept on the same diet for 4 more days, when the excretion of radio iron fell to a low level. Half of the animals were then put on the diet containing EDTA. Urine and feces were collected, and the 7th day after the radio iron had been administered blood samples and organs were removed for examination, as in experiments I and II. In this experiment the blood cells and plasma were separated. The results of this experiment are shown in Table II.

Results and Conclusions

The figures in Table I show clearly that EDTA interferes with the uptake of dietary iron. About two or three times as much radio iron was excreted in the EDTA-fed rats as in the controls, and the blood and organs of the controls accumulated up to 10 times as much as those of the EDTA-fed animals. This effect is more marked in experiment I, where the radio iron was fed to rats on a full stomach, which contained the full daily dose of EDTA. Apparently a sufficient amount of the EDTA is absorbed to increase urinary excretion. The general effect is that less iron is absorbed, and more of what is absorbed is excreted.

When EDTA was not fed until after the Fe^{59} had been absorbed, the iron appeared to be removed from the liver and to increase in the blood and the spleen, as shown in Table II. It would appear that when the sequestering agent enters the blood, the plasma iron is made non-utilizable, and more of it is excreted with the EDTA. Iron to replenish this loss probably comes from the liver. The high concentration in the blood plasma is probably a reflection of the presence of the Fe–EDTA complex, most of which will eventually be excreted. The high concentration of Fe^{59} in the spleen could be connected with the close association of the spleen with the vascular system, or possibly with phagocytic activity. It may be seen that the feeding of EDTA in experiment III did not cause an increase in the radioactivity of the feces. However, it is probable, from the results of experiments I and II, that there was a decreased absorption of dietary (non-radioactive) iron as the result of EDTA feeding. This decrease of available iron probably also had an influence on the apparent mobilization of body iron which was reflected in the changed radioactivities of the organs.

It is concluded that the presence of EDTA in the alimentary tract renders the iron less available for absorption. Some EDTA is, however, absorbed, and excreted by the kidneys as the iron complex. The ultimate effect is to render less of the absorbed iron available for metabolic purposes and to lower the metabolic stores.

References

1. Schwarzenbach, G. and Ackermann, H. Helv. Chim. Acta, **31**, 1029 (1948).
2. Krum, J. K. and Fellers, C. R. Food Technol. **6**, 103 (1952).
3. Smith, O. and Muneta, P. Am. Potato J. **31**, 404 (1954).
4. Greig, W. S. and Smith, O. Am. Potato J. **32**, 1 (1955).
5. Foreman, H., Vier, M., and Magee, M. J. Biol. Chem. **203**, 1045 (1953).
6. Foreman, H. and Trujillo, T. T. J. Lab. Clin. Med. **43**, 566 (1954).
7. Proescher, F. Proc. Soc. Exptl. Biol. Med. **76**, 619 (1951).
8. Spencer, H., Vankinscott, V., Lewin, I., and Laszlo, D. J. Clin. Invest. **31**, 1023 (1952).
9. Wishinsky, H., Weinberg, T., Prevost, E. M., Burgin, B., and Miller, M. J. J. Lab. Clin. Med. **42**, 550 (1953).
10. Schwarzenbach, G. and Heller, J. Helv. Chim. Acta, **34**, 576 (1951).
11. Foreman, H., Huff, R. L., Oda, J. M., and Garcia, J. Proc. Soc. Exptl. Biol. Med. **79**, 520 (1952).

THE TAC METHOD OF TRYPTIC ENZYME ACTIVITY DETERMINATION[1,2]

EDWARD RONWIN[3]

Abstract

A new, practical, and quantitative method for the determination of tryptic enzyme activity is reported. The method is based on measurements of the U.V. absorption of the complex formed between cupric ion and N^{α}-*p*-toluenesulphonyl-L-arginine, the latter being the product of the reaction between the tryptic enzymes (thrombin, trypsin, plasmin) and the corresponding methyl ester. Comparisons with the null point titration procedure demonstrate the new method's superiority when dealing with enzyme–substrate reactions in concentrated buffer solutions. Under certain conditions, it could also be superior in dilute buffer situations. The effects of pH and cupric ion concentration on complex formation are discussed along with other characteristics of the U.V. absorption curves of the complex.

Introduction

During the course of recent experiments it was noted that N^{α}-*p*-toluenesulphonyl-L-arginine (TA), which is the product of the reaction between tryptic enzymes (thrombin, plasmin, and trypsin) and the corresponding methyl ester (TAME), yielded a much increased U.V. absorption in the presence of Cu^{++} ion than seemed to be the case in the ion's absence. The possibilities that this effect might be utilized as a means of tryptic enzyme analysis, particularly under conditions where other known methods are either inapplicable or of dubious value, were immediately obvious. Preliminary tests confirmed that a U.V.-absorbing complex did indeed form. At this juncture, a search of the literature revealed that Budesinsky (1) had somewhat earlier noted the complex; however, he used a cumbersome chemical method for its detection which is completely unsuited for its quantitative analysis or for application to enzyme activity determinations. Following the preliminary tests, the complex was studied in greater detail, not from the physicochemical viewpoint of its specific properties as a complex (beyond the establishment of the physicochemical limits of its existence), but from the biochemical viewpoint as an additional, and it was hoped, practical tool in the analysis of tryptic enzyme activity. The results of that successful study are reported herein.

Materials and Methods

The new method described here, relying as it does on the U.V. absorption of a TA–Cu^{++} complex, will henceforth be referred to as the TAC method.[2] The currently accepted continuous titration (or null point titration) procedure for following the reaction between TAME and the tryptic enzymes will henceforth

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Contribution from the Department of Physiological Chemistry, University of Minnesota Medical School, Minneapolis, Minnesota. This work was done during the tenure of an Advanced Research Fellowship and Grant-in-aid from the American Heart Association.

[2] The name TAC is a monosyllabic rearrangement and abbreviation of the phrase Cu–TA complex.

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be referred to as the CT method. Comparisons between the TAC and CT methods are drawn in this study.

Enzymes, Substrate, and Enzyme–Substrate Reaction Product

Thrombin was a product of Parke, Davis & Co., lot No. 032001B. Trypsin was a twice-crystallized product and gift from Armour & Co. The activity of these enzymes is expressed in terms of the Th unit which has been previously defined (2, 3, 4). The substrate, TAME, was a product of H & M Chem. Co., Santa Monica, California; m.p. 147–149° no decomp. Calc. for the hydrochloride: C, 44.5%; H, 6.10%; N, 14.8%. Found: C, 44.42%; H, 6.09%; N, 14.6%. Although the enzyme reaction product, TA, can be made by the coupling of *p*-toluenesulphonyl chloride and arginine hydrochloride (5), it was found more convenient to prepare it from the methyl ester (TAME) using thrombin for the hydrolysis.

Preparation of N^{α}-p-Toluenesulphonyl-L-arginine.$3H_2O$ (TA) by Enzymatic Means

One gram of TAME was dissolved in 50 ml of water; 2 *N* NaOH was added dropwise until a slightly basic solution to universal indicator paper was achieved; 5000 NIH units of thrombin was then dissolved in 2 ml of distilled water and added in one step to the TAME solution. Following this, drops of 2 *N* NaOH were introduced as needed to keep the solution slightly alkaline. After 20 minutes a spontaneous precipitation of the product was observed. The supernatant was slightly alkaline; 0.2 *N* acetic acid was added until an acidic reaction was obtained. The precipitate was allowed to settle and then filtered off. The product was recrystallized from hot water. Crystallization in the filtered, refrigerator-cooled solution was induced by scratching; 0.5 g was obtained (yield, 51%); m.p. 255–256° decomp. (lit. 256–257° decomp. (5)). Calc. for the trihydrate: C, 40.8%; H, 6.8%; N, 14.7%. Found: C, 40.85%; H, 6.81%; N, 14.7%. The compound is somewhat soluble in cold water, not too soluble in alcohol, but very soluble in alkali.

Measurement of Enzyme Activity by the CT Method

The measurement of enzyme activity by the CT method has been described earlier (2). Two-milliliter reaction volumes and 10 to 20 μl of enzyme solutions were employed. Suitable controls were run.

Measurement of Enzyme Activity by the TAC Method

These measurements are relatively simple. The composition of the enzyme–substrate reaction solutions and the quantities of enzyme were either similar to or identical with those used in the runs measured by the CT method. At specific time intervals during the course of a reaction, 100-μl aliquots of the enzyme–substrate reaction solution to be tested were added to 10 ml of 0.001 *M* cupric acetate solution. The size of the aliquots of the test solution is so chosen that the concentration of TA in the 10 ml of cupric solution falls in the region of 10^{-5} to 10^{-4} *M*. Complex formation is exceedingly rapid and readings were made at room temperature in a Beckman DU spectrophotometer at 250 mμ using 1-cm silica cells. Concentrations of TA or the percentage of hydrolysis is then determined by use of a standard curve. For other determinations, such as the pH optimum for the formation of the complex, 0.01 *M* cupric chloride solutions were employed. The reasons for the switch to cupric acetate in the enzyme runs, as well as for the choice of 250 mμ for readings, will be considered in the Results and Discussion section.

Results and Discussion

Consideration of the U.V. Absorption Characteristics of the Complex, of TA, and of TAME

The data are given in Fig. 1. The curves for TA and TAME parallel each other closely. Increasing their concentration merely increases the heights of the curves but the general shapes are maintained. Over the range from pH 2 to 8, and in the same concentrations as in the situations in Fig. 1, the TA and TAME curves remain virtually unchanged. The curve for cupric ion (not shown in Fig. 1) is of similar shape but of much lower absorption magnitude.

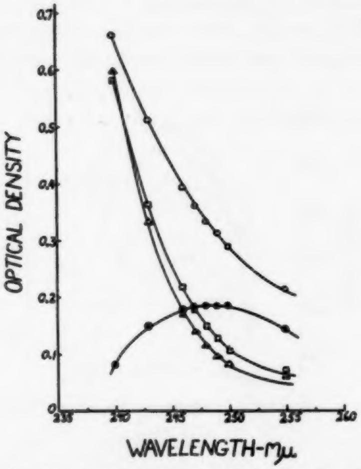

FIG. 1. U.V. absorption characteristics of the Cu–TA complex, of TA, and of TAME at pH 6.0; 1-cm matched silica cells were used. ○ 0.001 *M* $CuCl_2$ plus 0.0001 *M* TA (read against a 0.001 *M* $CuCl_2$ blank). □ 0.0001 *M* TA (read against water). △ 0.0001 *M* TAME with or without 0.0001 *M* $CuCl_2$ (read against either a water or cupric solution blank as the case indicated). Concentrations as high as 0.01 *M* $CuCl_2$ did not cause any Cu–TAME complex formation. ● Minimum absorption curve in this case for the Cu–TA complex; values obtained by subtracting TA alone curve from Cu–TA curve.

The cupric curve is concentration dependent but, unlike those for TA and TAME, is somewhat pH dependent; at a cupric ion concentration of 1×10^{-4} *M* and pH 5, its absorption vanishes. In the typical illustration of Fig. 1, the curve for the case of TAME plus cupric ion is identical with that for TAME alone (no points shown to avoid confusion of the diagram). In other cases the differences in values between Cu–TAME and TAME alone were either small negative or positive quantities. Thus, it may be reasonably concluded that TAME and Cu^{++} do not form a complex or at least one with an enhanced U.V. absorption over that of TAME alone. On the other hand, the curve for TA plus cupric ion (Fig. 1) is considerably higher than for TA alone and indicates the formation of a U.V.-absorbing complex. Subtraction of the values of the TA alone curve from those of the Cu–TA curve yields a curve for the minimum absorption of the complex which is represented by the line traced through the solid circles in Fig. 1. The peak appears from 248 to 250 mμ. The slight dip at 249 mμ may be significant since it has consistently appeared in numerous other cases. Because 250 mμ represents the wavelength at which the complex shows maximum or near maximum absorption, while that for TA alone is lower at 250 mμ than at 248 mμ, it was chosen as the operating wavelength for later studies in which the U.V. absorption of the complex is used to determine TA concentration and, thereby, enzyme activity. All the curves in Fig. 1 approach zero absorption asymptotically and reach this value (in these concentrations) in the region of 300 mμ.

Effect of pH on Cu–*TA Complex Formation*

At the concentrations of cupric ion and TA in Fig. 2, complex formation is restricted between pH 5 and 8. Over the absorption range shown, there is a gradual increase in complex formation from pH 5 to 6.5. Although the peak values for pH 6.5 are slightly lower than at 7 or 8, these differences are relatively small and it may be concluded that a constant yield of complex, at these concentrations, is obtained from pH 6.5 to 8.0. Above pH 8, the formation of cupric hydroxide makes it impossible to measure complex formation. The amount of complex formation is negligible at pH 5 and has been found to be nil at either pH 3 or pH 2. While cupric-chloride ion complex formation at these low pH's could contribute to interference with Cu–TA complexing, it is likely, considered with the fact that TAME apparently fails to complex with cupric ion, that Cu–TA complex formation requires a dissociated carboxyl group in

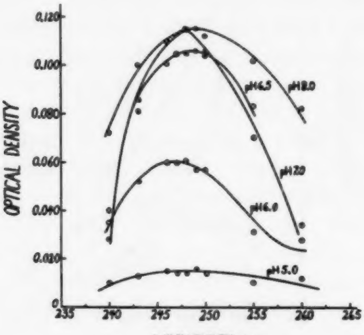

FIG. 2. Effect of pH on Cu–TA complex formation; 1-cm matched silica cells, 0.0001 *M* $CuCl_2$ plus 0.0001 *M* TA. Values for the complex are obtained by subtracting the sum of the absorption of the cupric solution alone plus that of TA alone, each read against a water blank adjusted to the proper pH, from the absorption of the Cu–TA solution also read against a water blank adjusted to the proper pH. At pH 2 or pH 3, no complex could be observed. Above pH 8, cupric hydroxide formation prevents detection or formation of the complex.

TA. However, using the values in Fig. 2, a plot of optical density versus pH at a specific optimum absorption wavelength, such as 250 mμ, results in a sigmoid curve (not given in order to conserve space), which is essentially a spectrophotometric titration of TA by cupric ion and has a pK at approximately 6 which is indicative that more than simple co-ordination with the carboxyl group of TA is involved.

The level of permissible cupric ion concentration is also a function of pH. At pH 7, raising the concentration of the ion from 0.0001 M to 0.0003 M yielded no increased Cu–TA complex formation; furthermore, the latter proved an upper limit to effective cupric ion concentration at this pH. Probably, as at pH 8, cupric hydroxide formation became significant and interfered with Cu–TA formation.

Effect of Cupric Ion Concentration on Cu–*TA Complex Formation*

Although pH 6 is not an optimum for complex formation, it is a value at which effective and significant measurements can be obtained and where formation of cupric hydroxide and possibly other side effects are minimized. Therefore, it was chosen as the pH at which to determine the effect of Cu^{++} concentration on the degree of complex formation. The data are presented in Fig. 3. In the course of these experiments it was found that a concentration of 0.02 M cupric ion was too dense as a blank; 0.01 M cupric ion is apparently close to an operative limit. However, the absorption of the complex resulting from 0.01 M Cu^{++} plus 0.0001 M TA was too strong to be read at 240 mμ against the 0.01 M Cu^{++} blank, though it permits readings at higher wave-

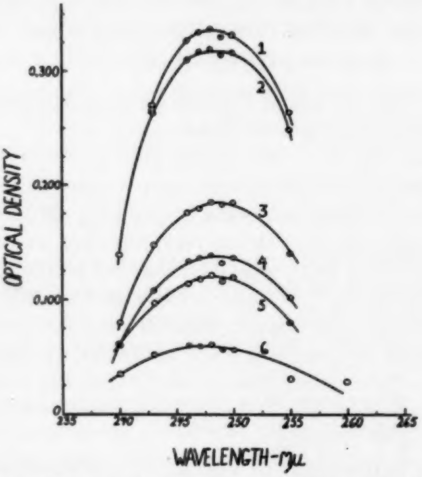

FIG. 3. Effect of cupric ion concentration on Cu–TA complex formation at pH 6.0; 0.0001 M TA, 1-cm matched silica cells. Curve 1, 0.01 M $CuCl_2$; curve 2, 0.003 M $CuCl_2$; curve 3, 0.001 M $CuCl_2$; curve 4, 0.0007 M $CuCl_2$; curve 5, 0.0003 M $CuCl_2$; curve 6, 0.0001 M $CuCl_2$. All Cu–TA readings were made against the appropriate cupric blank from which values for that of TA alone were subtracted to obtain the values given. A concentration of 0.02 M $CuCl_2$ proved too dense from 240 to 260 mμ.

lengths. Under the conditions in Fig. 3, there is a gradual increase in complex formation as cupric ion concentration is increased over the entire absorption wavelength region. If the data of Fig. 3 are also employed to plot the O.D. versus the concentration of Cu^{++} at a specific optimum absorption wavelength, such as at 250 mμ, a curve is obtained (not given in order to conserve space) which indicates that maximum optical densities are obtained when Cu^{++} concentration is 30 times that of TA. However, the data of such a curve, as well as of Fig. 3, do not appear to be consistent with any simple ratio or relationship between Cu^{++} and TA. It seems improbable that the 10:1 ratio of Cu:TA reported by Budesinsky (1) is correct.

As mentioned earlier, the slight dip in complex absorption at 249 mμ is observed in all the curves of Fig. 3 and it appears likely that it represents an actual situation rather than an experimental artifact.

Investigation of Possible Complexing between Ni^{++} *and* Ca^{++} *and either TA or TAME*

Space considerations make it unnecessary to present the pertinent data, particularly as the results of the investigation allow brief summation. At either pH 6 or pH 8, neither Ni^{++} nor Ca^{++} form demonstrable U.V.-absorbing complexes with TA or TAME in the region from 235 to 310 mμ. This observation is germane to earlier work by this investigator, especially to some recently published material (4) in which the interaction of either thrombin or trypsin with these ions in the presence of both TA or TAME is considered.

Application of the TAC Method to Tryptic Enzyme Measurement: Preliminary Considerations and the Standard Curve

Having established the U.V. absorption characteristics of the complex and studied both the effects of concentration and pH upon it, attention was turned to its possible use as a means of following tryptic enzyme activity. In the earlier studies, cupric chloride was used exclusively; however, as the possible result of hydrolysis by virtue of its weak base – strong acid character, solutions of this salt showed slow changes in U.V. absorption with time and attempts to apply the method to tryptic enzyme activity measurement met with repeated failure. Resort was then made to cupric acetate which led to the successful results reported below. One interesting observation is that the yield of complex, at a constant TA concentration, is somewhat higher with 0.001 *M* cupric acetate than with 0.01 *M* cupric chloride.

A standard curve is presented in Fig. 4. It was constructed with aliquots from standard solutions simulating enzyme reaction conditions both in dilute buffer (0.027 *M* veronal) and concentrated buffer (0.2 *M* tris), and containing varying ratios of TA and TAME with 100% of each represented by a concentration of 0.01 *M*. These standard solutions were at pH 7.8; 100-μl aliquots of these standard solutions, representing various percentages of hydrolysis of TAME, were withdrawn and pipetted into 10 ml of 0.001 *M* cupric acetate solution (pH 5.9). This raised the pH of the cupric acetate solutions to 6.4. Complex formation is virtually instantaneous and the cupric acetate solutions were read at 250 mμ against a cupric acetate blank containing the proper

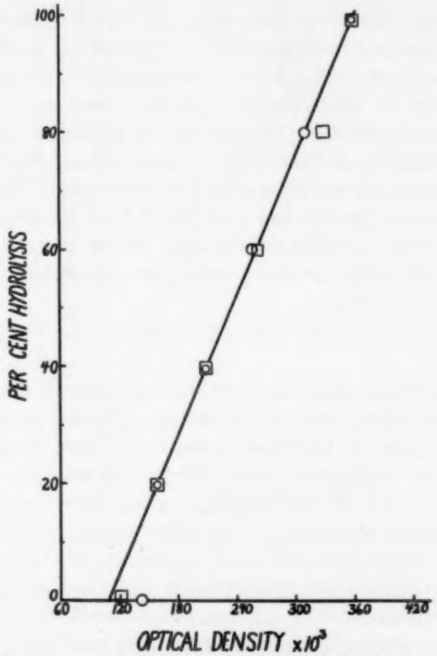

FIG. 4. Standard curve on Cu–TA complex simulating enzyme reaction conditions. Zero per cent hydrolysis corresponds to 0.01 *M* TAME in the enzyme–substrate reaction mixture, while 100% hydrolysis corresponds to 0.01 *M* TA in the enzyme–substrate reaction mixture; 100-μl aliquots of these solutions and of the other standard solutions containing varying ratios of TA to TAME (but no enzyme) were pipetted into 10 ml of 0.001 *M* cupric acetate and read against a cupric acetate blank containing the appropriate buffer at 250 mμ using 1-cm matched silica cells. ○ 0.027 *M* veronal buffer; □ 0.2 *M* tris buffer (blank had an optical density of 0.150 at 1.9 slit width).

amount of buffer. Gratifyingly, the two situations (dilute and concentrated buffer) yielded four points in common with little other variation and the resultant curves are reasonably represented by a single straight line which is in agreement with the Beer–Lambert relationship. This is all the more interesting since the blank which contained a 100-μl aliquot of 0.2 *M* tris buffer could not be brought to zero on the optical density scale despite the use of maximum slit width. Under these conditions, the lowest value on the optical density scale at which the spectrophotometer could be brought to zero was 0.150. From test solution readings in this case, this blank value was subtracted to obtain those given in Fig. 4. Although the possibility was not pursued, it is probable that the tris molecule forms a complex with cupric ion not unlike that between ammonia and cupric ion, since addition of the aliquots containing tris buffer to the cupric acetate solution results in the immediate impartation of a decidedly blue hue to the cupric acetate solution. Also, the increased opacity of the blanks containing tris buffer as compared with those in which it is absent, considered along with the fact that tris buffer alone in 50 times the concentration has practically no absorption over the range from 235 to 290 mμ, points to Cu–tris complex formation. However, the results in Fig. 4 indicate that the possible Cu–tris complexdoes not interfere with the formation of the Cu–TA complex. Veronal buffer does not form a detectable complex with cupric ion. The switch from veronal buffer in dilute buffer situations to tris in concentrated buffer situations was necessitated by the limited solubility of veronal buffer below pH 8. One shortcoming of the standard curve is that the values in the region from 0 to 20% hydrolysis are somewhat less trustworthy than those above 20% hydrolysis. This is due to the fact that the TAME absorption is quite significant in the former region while the changes due to increased TA in this same region are still sufficiently small to make the values less reliable than in the region above 20% hydrolysis.

Comparison between the TAC and CT Methods for Trypsin Activity Determinations

For the TAC method, the procedure is identical with that used in obtaining the standard curve, except that the blanks are adjusted to include the appropriate quantity of enzyme. It had been earlier found that the quantities of trypsin used were devoid of absorption from 240 to 255 mμ. In concentrated buffer situations, such as at 0.2 M tris, the pH, as gauged by the galvanometer needle of a Beckman model G pH meter, is virtually constant throughout the reaction. Thus, test aliquots in concentrated buffer situations are taken at periods decided upon at will and the complex is developed and read as discussed above. Of course, as the Beckman meter galvanometer needle is virtually constant in concentrated buffer during an enzyme–substrate reaction under the conditions used here, the CT method is inoperative and the TAC method is on this account superior. However, in dilute buffer situations, such as in 0.027 M veronal, the galvanometer needle readily responds to the pH changes caused by the TA released during enzyme hydrolysis and provides the basis for the CT method. Thus, in dilute buffer situations, when the TAC method is being applied to obtain values for comparison with the CT method, base was added during the course of the enzyme–substrate reaction to keep the pH constant. In the TAC method in dilute buffer situations, the base added-time values during the early course of any reaction will correspond to a regular CT method run, but as more aliquots are removed this correspondence will be lost. The need to add base when working in dilute buffer situations in these pure systems (thereby performing a veritable CT determination) demonstrates the superiority of the CT method over the TAC method in dilute buffer situations.

Figure 5 contains the pertinent data. The percentage of hydrolysis in the TAC method is simply obtained by reading the value from the corresponding absorption of the test solution at 250 mμ as given in Fig. 4. This value is plotted against the time at which the aliquot, withdrawn from the enzyme–substrate reaction solution, was pipetted into the 10-ml cupric acetate solution. While some points show wide variation, the results demonstrate that the values from two runs, obtained for the hydrolysis of TAME by trypsin using the TAC method in dilute buffer situations, vary by less than 5% over much of the reaction course and is nowhere greater than 7% as compared with the values obtained by the CT method. Since, as noted above, the CT method is

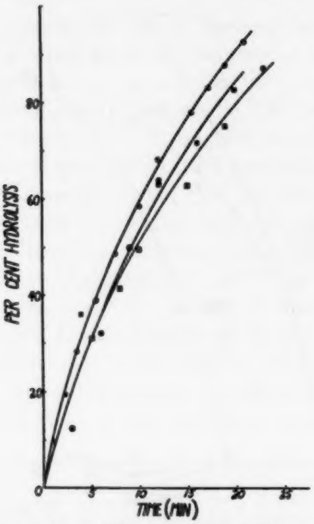

FIG. 5. Comparison between the TAC and CT methods for trypsin activity determinations. The procedures for the CT and TAC methods are described in the text. Enzyme reaction conditions: 0.01 *M* TAME initial substrate concentration, 38° C, pH 8.0, 2-ml reaction volumes, and either 0.027 *M* veronal (dilute buffer) or 0.2 *M* tris (concentrated buffer). Trypsin concentration: 3.41 Th units/ml. ○ CT, dilute buffer; ● TAC, dilute buffer; ■ TAC, dilute buffer; □ TAC, concentrated buffer.

inoperative in concentrated buffer, no comparison between the CT and TAC methods is possible in these situations; however, a comparison between the TAC method in dilute buffer and the TAC method in concentrated buffer situations shows that over 60% of the course of the enzyme–substrate reaction insignificant variation occurs under the conditions used here. Deviations over the final stages of the reaction are small (5% from dilute buffer–TAC and 10% from dilute buffer–CT) and are likely due to the known repressive effects of high ionic strengths.

The results permit the reasonable conclusion that trypsin activity can be satisfactorily followed in both concentrated and dilute buffer situations using the TAC method; with TAC necessitated in concentrated buffer and CT preferred in dilute buffer situations.

Comparison between the TAC and CT Methods for Thrombin Activity Determinations

Much the same considerations which applied to the case of trypsin, such as the need to add base to the enzyme–substrate reaction solutions in applying the TAC method in dilute buffer situations, apply here. So, too, the procedures are identical. The data are given in Fig. 6. The B abscissa corresponds to the runs in dilute buffer; the A abscissa to those in concentrated buffer situations. Separation of the curves as in Fig. 6 was desirable to avoid confusion of the diagram. In dilute buffer situations, Fig. 6, B, the correspondence between both methods is excellent. In Fig. 6, A, a comparison is presented using the

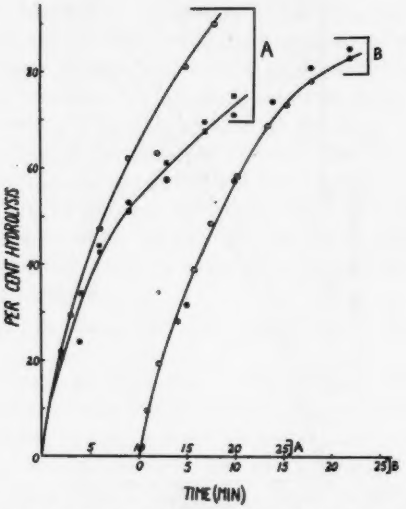

FIG. 6. Comparison between the TAC and CT methods for thrombin activity determinations. The procedures for the CT and TAC methods are described in the text. Enzyme reaction conditions: 0.01 *M* TAME initial substrate concentration, 38° C, pH 8.0, 2-ml reaction volumes, and either 0.027 M veronal (dilute buffer) or 0.2 *M* tris (concentrated buffer). The runs in section A had concentrated buffer and the TAC method was used: ○ thrombin concentration, 3.76 Th units/ml; ● and ■ thrombin concentration, 3.41 Th units/ml. The runs in section B had dilute buffer and a thrombin concentration of 3.41 Th units/ml: ○ CT method; ● TAC method.

TAC method for concentrated buffer situations at two different enzyme concentrations. Again, the CT method is inoperative in concentrated buffer and no comparison with TAC can be made. The ratio of the lower-to-higher enzyme concentration was 0.90 and that for the two curves is 0.87 over most of the reaction course. For the case of the lower enzyme concentration in the concentrated buffer situations, Fig. 6, A, there is close correspondence of the two separate runs reported and both are well represented by a single curve. If this curve were placed in comparison with that of Fig. 6, B (where the same enzyme concentration was used), it would be identical over 50% of the course of the reaction; the rate slows in the later stages as the probable result of the known inhibition of high ionic strengths on thrombin activity (2). It is of additional interest that the Cu–TA complex is stable over a period of at least 24 hours. Hence, test aliquots on a series of enzyme–substrate runs can be collected at one time and read at a more convenient hour.

Conclusion

A new, practical, and quantitative method for tryptic enzyme activity measurement has been developed. Certainly, for enzyme–substrate reactions in concentrated buffer, where the CT method is inoperative, there is no question of the TAC method's superiority. In dilute buffer situations, CT is the method of choice because the need to add base to maintain a constant pH in the TAC method is a veritable application of the CT method; however, it is conceivable that in some dilute buffer situations such factors as high temperature, high enzyme, or chemical agents and ions capable of interfering with the glass electrode response would mediate in favor of the TAC method. The previously emphasized similarity between plasmin, trypsin, and thrombin (2) makes it probable that plasmin activity can be likewise measured by the TAC method.

Either in print (2) or in other forms of comment, this investigator has repeatedly stressed the fact that the clotting test is completely unsatisfactory as a quantitative method. Therefore, no correlation between it and the TAC method was attempted. In pure systems, either CT or TAC now enables us to make quantitative measurements of thrombin and trypsin (presumably plasmin, too) activity. With blood, plasma, or serum, we are still dependent upon the clotting test; however, it is anticipated that the TAC method, while surely encountering obstacles, will eventually be found suitable for the measurement of tryptic enzyme activities in biological and related fluids.

References

1. Budesinsky, B. Chem. listy, **50**, 1236 (1956).
2. Ronwin, E. Can. J. Biochem. and Physiol. **35**, 743 (1957).
3. Ronwin, E. Can. J. Biochem. and Physiol. **36**, 75 (1958).
4. Ronwin, E. Biochim. et Biophys. Acta, **33**, 326 (1959).
5. Bergmann, M., Fruton, J. S., and Pollok, H. J. Biol. Chem. **127**, 643 (1939).

CARBOHYDRATE UTILIZATION BY CHICK EMBRYONIC HEART CULTURES[1]

JOSEPH F. MORGAN AND HELEN J. MORTON

Abstract

Freshly explanted chick embryonic heart fragments were cultivated in completely synthetic media. Survival of such cultures in the complete medium, containing glucose, was established at approximately 35 to 40 days, while in the absence of carbohydrate the cultures died within 3 to 5 days. Survival was considered to be a more physiological measurement than rapid cell multiplication for normal tissues and was adopted as the criterion for all experiments reported. Fifty-two compounds were tested for their ability to replace glucose, as the sole carbohydrate, in this system. Of these, seven (mannose, fructose, galactose, β-glucose, maltose, glucose-1-phosphate, and glucose-6-phosphate) replaced glucose completely. Five others (sorbitol, alpha-methyl-D-glucoside, turanose, dextrin, and fructose-6-phosphate) were partially active. The remainder were negative. Comparison is made of the present results with those obtained by other workers using malignant cells in the presence of serum–enzymes. The present results suggest that the ability to replace glucose decreases progressively as compounds down the Embden–Meyerhof pathway are tested.

The early tissue culture literature contains many reports on the carbohydrate metabolism of cells in vitro (1, 2, 3, 4). In general, these studies were mainly concerned with relative rates of glucose utilization and with the extent of conversion of added glucose to lactic acid. With the establishment of many permanent cell lines in tissue culture, considerable emphasis has been placed on the ability of cell strains to utilize various carbohydrates, in the hope that different requirements might be established between normal and malignant cells. Two extensive surveys of carbohydrate utilization by established cell strains of human malignant origin have recently been reported (5, 6).

Up to the present time, investigations on the carbohydrate requirement of cell cultures have been carried out in complex media containing unknown nutritional factors. Most such media have contained dialyzed serum, which, by virtue of its ability to hydrolyze di- and poly-saccharides, must inevitably yield equivocal results. This hazard has been recognized by some workers (6, 7) but ignored by others (5). Since the carbohydrase activity of serum varies with the type used (8), it seemed essential to determine the carbohydrate requirements of isolated cells in completely synthetic media, free from such variables.

The established cell strains commonly used in tissue culture research are mainly of malignant origin, but those of normal tissue origin have shown an alteration towards malignant characteristics in both morphology (9, 10) and metabolism (11, 12) after prolonged cultivation in vitro. It is questionable, therefore, whether any established cell line can be regarded as truly representative of normal cells. Since there is considerable evidence that the carbohydrate metabolism of malignant cells differs, at least quantitatively, from that of normal tissues (13, 14), studies on normal cultures appear urgently needed to provide a baseline for comparison with the malignant cell types.

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Contribution from the Laboratory of Hygiene, Department of National Health and Welfare, Ottawa, Ontario.

The present study was undertaken to determine the carbohydrate requirement of a freshly explanted normal tissue in a completely synthetic medium free from unknown nutritional factors. The ability of such chick embryonic heart cultures to utilize a variety of carbohydrates forms the basis of this communication.

Materials and Methods

Tissue Culture Techniques

The main techniques used in preparing experimental cultures have been described previously (15, 16). In the present study, the cells were depleted of carbohydrate and other nutritional reserves before the start of the experiment. Since cells depleted of carbohydrates and protein are particularly sensitive to shock (6, 17), the depletion method used previously (17) was modified slightly. The freshly explanted tissues were placed in roller tubes and allowed to lie in a horizontal position for 2 days while covered by Hanks' balanced salt solution (18) from which glucose had been omitted. If this depletion period was extended to 3 days, the tissues were so severely damaged that they could not be revived by the complete medium, but died within 6 days regardless of subsequent treatment. Also, in salt solution without glucose, outgrowth from the tissue fragments was very slight. Since this is the only means by which the tissues adhere to the glass, many fragments failed to adhere. This difficulty was overcome by reducing the volume of medium to 0.5 ml per roller tube. These conditions were selected as the most severe which could be used and still yield uniform experimental groups of cultures. Other techniques were used exactly as described previously (17).

After the depletion period, the cultures were examined microscopically, divided into comparable groups, and supplied with 1.5 ml of experimental medium per tube. The media were replaced twice weekly, the cultures examined three times weekly until death, and average survival times calculated for each test solution. All figures reported represent an average of several experiments, each containing 10 to 12 individual cultures per solution.

Experimental Media

The positive control medium in these experiments, M 150, has been described previously (16, 19). The negative control medium, M 1232, contained all the ingredients of M 150 with the exception of the carbohydrates glucose, ribose, and deoxyribose. Experimental media were prepared by adding the required amount of a given carbohydrate to the sugar-free medium. When high concentrations of glucose were tested the sodium chloride content of the medium was adjusted to maintain isotonicity. All solutions were sterilized by filtration through ultrafine fritted glass filters. All carbohydrates used in these experiments were tested for purity by paper chromatography.

Sensitivity of the Test System

As indicated above, cultures not supplied with glucose were all dead 6 days after explantation, i.e., within 4 days of the start of the experiment. With 323 cultures used as negative controls in these studies, the average survival time was 3.5 days. Survivals of 8 days from the start of the experiment are, accordingly, highly significant despite their small numerical value. By this depletion technique, it is possible to distinguish between sugars which can partially replace glucose and those which are completely ineffective. Survival time of the positive controls in the complete medium, M 150, ranged between 35 and 40 days, with an average value of 37.3 days.

Results

Ability of Various Carbohydrates to Replace Glucose

The carbohydrates tested for their ability to replace glucose are summarized in Table I. All compounds were tested at a level of 1.0 g of carbohydrate per liter of M 1232, a concentration equivalent to the glucose content of medium M 150. It can be seen (Table I) that mannose, fructose, galactose, β-glucose, maltose, glucose-1-phosphate, and glucose-6-phosphate were equal or nearly equal to glucose in their ability to support cell survival. Turanose, sorbitol, methyl-D-glucoside, and dextrin showed moderate activity. Fructose-6-phosphate showed slight activity. The remaining 40 other compounds tested were completely ineffective.

TABLE I

Ability of various compounds to replace glucose in the nutrition of chick embryonic heart cultures*

Carbohydrate	Days†	Carbohydrate	Days†	Carbohydrate	Days†
Controls + glucose	37.3	Methylglucosides		Fructose-1,6-PO$_4$	3.2
− glucose	3.5	α-Methyl-D-glucoside	*12.8*	Ribose-5-PO$_4$	4.3
		Methyl-D-mannoside	4.0	Na phosphoglycerate	3.6
Pentoses				β-Glycerophosphate	3.0
Lyxose	4.1	Disaccharides			
Xylose	3.7	Maltose	*31.4*	Citric acid cycle components	
Ribose	3.5	Turanose	*24.8*	Na pyruvate	4.0
Deoxyribose	3.0	Trehalose	2.5	Na citrate	2.9
L-Arabinose	2.5	Cellobiose	3.9	Na succinate	2.8
D-Arabinose	3.9	Lactose	2.0	Na fumarate	2.5
		Sucrose	4.0	Na malate	3.1
Hexoses		Melibiose	4.0		
Mannose	*36.0*			Oxidized sugars	
Fructose	*32.3*	Trisaccharides		Glucuronic acid	4.0
Galactose	*32.0*	Raffinose	2.4	Glucuronolactone	4.0
β-Glucose	*35.5*	Melezitose	3.5	Galacturonic acid	3.5
Sorbose	2.6			Phosphogluconic acid	4.1
		Polysaccharides			
Alcohols		Dextrin	*10.5*	Miscellaneous	
Sorbitol	*13.3*	Glycogen	4.0	Aesculin	4.1
Mannitol	3.1	Inulin	3.7	Glycerol	2.8
Arabitol	3.1	Starch	4.0	Na lactate	4.0
Dulcitol	3.5				
Erythritol	3.7	Phosphorylated sugars, etc.			
		Glucose-1-PO$_4$	*33.7*		
Methyl sugars		Glucose-6-PO$_4$	*37.5*		
Fucose	3.2	Fructose-6-PO$_4$	*8.0*		
Rhamnose	2.7				

*All compounds added to sugar-free M 1232 at the level of 1 g per liter.

†Days from the start of the experiment. Each figure is the average survival of 20 to 40 individual cultures.

Since these results were obtained with the arbitrary concentration of 1.0 g per liter, which might not necessarily be the optimum level, it was decided to test the effect of the addition of graded levels of the active compounds to the sugar-free medium.

Effect of Graded Levels of Monosaccharides on Culture Survival

The sugar-free medium (M 1232) was prepared and to it were added increasing concentrations of either glucose, mannose, fructose, or β-glucose. The average survival of cultures in each solution was determined and the results are summarized in Fig. 1.

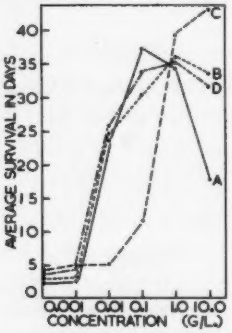


FIG. 1. Effect of graded levels of monosaccharides on survival of chick embryonic heart cultures. A, glucose; B, mannose; C, fructose; D, β-glucose.

It is apparent that glucose, mannose, and β-glucose have similar effects on culture survival. The addition of increasing amounts of these compounds increases the survival of chick embryonic heart fragments, with maximum effect at either 0.1 or 1.0 g per liter. The addition of 10.0 g per liter of any of these sugars is inhibitory, particularly in the case of glucose. The addition of graded levels of fructose to the sugar-free medium caused a distinctly different type of response from that effected by the other monosaccharides. Considerably higher levels of fructose were required to show any effect on cell survival and no inhibition was found, even at a concentration of 10.0 g per liter.

Variable results were obtained when galactose was tested as the sole carbohydrate in the medium, as had been reported by other workers (5, 6). Since chromatographic analysis revealed considerable amounts of contaminants in certain preparations of galactose, it was decided to compare the effects of different preparations on cell survival. The preparations tested are described in Table II. The response of chick embryo heart cultures to these preparations is shown in Fig. 2. It can be seen that preparations A and B produced almost identical results. Preparation C permitted a survival nearly equal to that supported by A and B, but the shape of the response curve was distinctly different, since maximum survival was reached at a lower concentration than with the other two preparations. Preparation D produced a completely different type of response and a much shorter culture survival.

By comparison of Fig. 2 and Table II, it can be seen that the type and amount of contaminant are important to cell survival. The trace contaminant in preparations C and D was suspected to be glucuronic acid from its color reactions on paper chromatograms, but was not positively identified. Since the

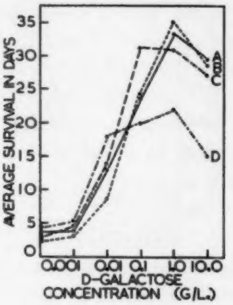


FIG. 2. Effect of different preparations of galactose on cell survival. A, impure commercial preparation; B, pure commercial preparation with added pyruvate; C, pure commercial preparation; D, commercial preparation freed of glucose by treatment with yeast and recrystallization.

preparation with the higher amount of this contaminant was less effective in supporting culture survival, it would appear that inhibition of galactose utilization might have occurred. The possible role of glucuronic and galacturonic acids as inhibitors of galactose utilization in tissue cultures is now under investigation. It has been reported (5) that the addition of pyruvate, in the presence of galactose, reduces the variability of the tissue culture response to this sugar. Comparison of curves A and B with curve C of Fig. 2 suggests that these earlier results (5) may have been due to a variable pyruvate or glucose contamination in the galactose employed.

TABLE II

Analysis of galactose samples employed

Preparation	Description	Chromatographic results
A	Commercial, untreated	Contained approximately 50% glucose plus other contaminants
D*	Commercial, purified by yeast treatment to remove glucose, and repeated recrystallization	Glucose-free, contained a trace of a single acidic non-reducing compound
C	Commercial, labelled as "chromatographically pure"	Glucose-free, contained the same contaminant as sample D but in smaller amount
B	Preparation C with 1.0 mmolar pyruvate added.	—

*We are indebted to Dr. D. M. L. Michener, Department of Biochemistry, University of Toronto, for this material.

Effect of Graded Levels of Disaccharides on Culture Survival

The two disaccharides, maltose and turanose, which showed ability to replace glucose at the 1.0 g per liter level, were added, individually, to M 1232 at concentrations ranging from 1.0 mg per liter to 10.0 g per liter. Two previously negative disaccharides, sucrose and lactose, were also tested over the same concentrations because of their known importance in biological systems. The results of these experiments are presented in Fig. 3. It is apparent that sucrose (line B) and lactose (line D) are not utilized for glucose replacement at any

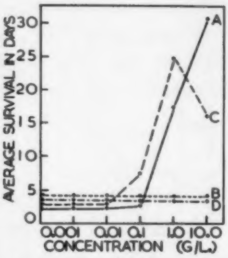
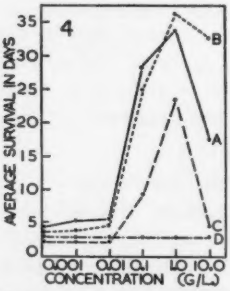


FIG. 3. Effect of graded levels of disaccharides on survival of chick heart cultures. A, maltose; B, sucrose; C, turanose; D, lactose.

concentration. This is in agreement with the findings of other workers (3, 4, 5, 6, 7). Maltose (line A), in contrast to the monosaccharides, is ineffectual until a level of at least 1.0 g per liter is present, but at higher concentrations supports a survival equivalent to the 1.0 g per liter level of glucose (Fig. 1, line A). Turanose, on the other hand, showed its optimum effect at 1.0 g per liter and was less effective at higher concentrations (line C).

Effect of Graded Levels of Phosphorylated Sugars on Culture Survival

Graded levels of four phosphorylated sugars were incorporated in M 1232, culture survival determined in each solution, and the results summarized in Fig. 4. It can be seen that the cells are able to utilize glucose-1-phosphate and glucose-6-phosphate (lines A and B) equally well. Fructose-6-phosphate (line C) is less effective than the glucose phosphates at 1.0 g per liter and shows a more marked toxicity at 10.0 g per liter. Fructose diphosphate (line D) was completely ineffective at all concentrations tested. It would appear from these results that there is decreasing efficiency of utilization as compounds farther down the Embden–Meyerhof pathway are tested. From the data of Table I it can also be seen that other compounds below fructose diphosphate on this pathway were consistently negative.



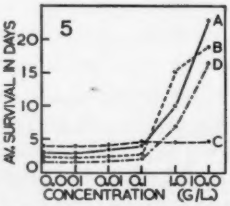


FIG. 4. Effect of graded levels of phosphorylated sugars on survival of chick heart cultures. A, glucose-1-phosphate; B, glucose-6-phosphate; C, fructose-6-phosphate; D, fructose-1,6-diphosphate.

FIG. 5. Effect of graded levels of various carbohydrates on survival of chick heart cultures. A, dextrin; B, sorbitol; C, mannitol; D, alpha-methyl-D-glucoside.

Utilization of Related Compounds by Tissue Cultures

From the data of Table I, it can be seen that glucose, maltose, and dextrin are progressively less efficient in supporting cell survival. This observation is consistent with molecular size and possible reduced cell permeability to the larger molecules. It was, therefore, considered of interest to determine the response of the cultures to graded levels of dextrin incorporated in the sugar-free medium. In Fig. 5, line A, it can be seen that even at high levels dextrin does not support the survival of cells for periods as long as those supported by maltose (Fig. 3, line A) or glucose (Fig. 1, line A). The testing of higher levels of dextrin proved impossible because of the limited solubility of the compound in the test media. The possibility that contamination of the dextrin with trace amounts of glucose may have been responsible for the observed activity could not be entirely eliminated. It was rendered unlikely, however, by paper chromatographic tests which showed only negligible amounts of glucose.

Only one sugar alcohol, sorbitol, showed any ability to replace glucose (Table I). It was, accordingly, tested over a wide range of concentrations. Mannitol was also tested because its corresponding sugar had shown activity, especially at high concentrations. In Fig. 5, line B, sorbitol is shown to have a moderate ability to replace glucose. Its ability to support culture survival increases with increasing concentration. Mannitol (line C) is completely ineffective. The special position of sorbitol is undoubtedly due to the existence of pathways for its conversion to either fructose (20) or glucose (21). There is no indication at present of similar pathways in animal tissues for other sugar alcohols.

In Fig. 5, line D, is shown the response curve obtained when graded levels of alpha-methyl-D-glucoside are added to medium M 1232 and supplied to cultures. It is apparent that the chick embryonic heart cells can split this alpha-glucoside, although at a slow rate. The survival obtained with 10.0 g per liter of the glucoside is roughly equivalent to that obtained with 5.0 mg per liter of glucose (Fig. 1, line A).

Discussion

Because of its role as the energy source for biological processes, carbohydrate metabolism holds a unique position in all studies of living cells. This is particularly true of investigations of the distinguishing characteristics of malignant cells and the carcinogenic process (13, 14, 22, 23). Tissue culture, with its facilities for long-term cultivation of cells and the possibility of observing them during a period of changing metabolic patterns (24–26), offers a particularly useful experimental system. In order to use this technique for comparative studies of normal and malignant cells, it is essential to have information on the carbohydrate utilization by truly normal cells. It is also essential that these results be obtained in completely synthetic media, free from contaminating enzymes and unknown nutritional factors. The present survey of the carbohydrate requirements of chick embryo heart muscle, based on more than 5000 individual cultures, is the first study specifically designed to satisfy both these conditions.

The results have shown that a variety of monosaccharides can be utilized in place of glucose but that the type of response varies with different sugars. Although galactose, mannose, fructose, and β-glucose can all replace glucose in this system (Figs. 1 and 2), fructose is distinctive in that increasing cell survival is obtained with increasing concentration. The other monosaccharides showed varying degrees of inhibition at the highest concentration tested (10.0 g per liter). This finding is in agreement with the earlier results of Harris and Kutsky (7) and Eagle and associates (5), who showed that fructose utilization is less efficient than that of glucose. The most plausible explanation for this finding would be reduced cellular permeability to fructose. On the other hand, intracellular accumulation of glucose has been shown to occur under conditions which did not produce accumulation of fructose (27). If similar mechanisms were operating in the present culture system, either glucose or its products could cause a metabolic imbalance by the mechanism of feedback inhibition (28). Assuming that fructose enters entirely and directly into the Embden–Meyerhof scheme, the difference between it and glucose could be accounted for entirely by a slower rate of fructose uptake. On the other hand, decreased rate of fructose uptake could prevent the accumulation of excess products able to activate feedback inhibition. Either of these would account for the decreased efficiency of fructose and for the lack of toxicity at the highest level.

The reaction of the two disaccharides, maltose and turanose, is in distinct contrast to that of their constituent sugars. Maltose (glucose–glucose) resembles fructose in that it supports increasing survival with increasing concentration. It is possible that this is a reflection of either a slow rate of hydrolysis or limited cell permeability and resulting failure to activate feedback mechanisms. Turanose consists of glucose–fructose and shows a pattern of behavior intermediate between that of its constituent sugars.

It is also possible that fructose occupies a special position among the hexoses. In rat liver slices, high carbohydrate feeding has been shown to cause impaired metabolism of glucose but not of fructose (29). This observation is comparable to the results obtained with high concentrations of hexoses in the present system. It has also been shown that fructose causes an induced enzyme type of response in the enzymes concerned with the conversion of fructose to glucose (30, 31). Although this effect was seen only in liver, heart was not among the tissues tested. The decreased efficiency of fructose in the present system would not suggest enzyme induction, but the conditions may not have been optimum. The behavior of turanose (Fig. 3) may possibly be explained by mechanisms similar to that of fructose.

The response curves obtained with phosphorylated sugars (Fig. 4) indicate that metabolic balances may also distinguish the metabolism of the hexose esters. In the first place, although glucose-1-phosphate and glucose-6-phosphate support almost identical culture survival times at the optimum level of 1.0 g per liter, the former is much more inhibitory at the 10.0 g per liter level. This may be due to a disturbance of metabolism centered on uridine diphosphoglucose (UDPG) as found in other systems (32, 33). Excess glucose-1-phosphate has been shown to cause accumulation of UDPG and galactose-1-phosphate by its effect on UDPG-pyrophosphorylase (33).

Chick embryo heart cells apparently distinguish between glucose-6-phosphate and fructose-6-phosphate in the same manner as they distinguish between the corresponding sugars (compare Figs. 1 and 4). Larger amounts of either fructose or fructose-6-phosphate are required to support a survival equivalent to that obtained with glucose or its ester. Recent studies have now shown that the failure of fructose-1,6-diphosphate is due to a lack of phosphate acceptors (34). Similar imbalance between essential cofactors is probably also responsible for some of the negative results reported and investigations of individual systems are in progress to elucidate these mechanisms.

Of the compounds known to be converted to glucose via uridine diphosphoglucose, only galactose was active in the tissue culture system. Since these compounds were all assayed in the absence of glucose or added pyrimidine nucleotides, this finding may reflect the failure of the cells to synthesize UDPG rather than the absence of transuridylases or specific enzymes such as sucrase or trehalase. It is interesting to note that Eagle and associates (5), using a medium containing dialyzed serum, found trehalose to be active. The difference in results may be due to tissue specificity, or to enzyme activity of the medium. Galactose has also been shown to be oxidized directly in liver (35), without the intervention of UDPG. Studies to date in tissue culture do not indicate which specific mechanism of galactose utilization functions in chick embryo heart. However, comparison of different samples of galactose (Table II) suggests that competitive or 'symbiotic' systems may exist when other products are present as trace contaminants in galactose samples. These impurities may have been responsible for the variable results reported earlier (5) and for the failure of cells to grow in the presence of galactose once they were past the logarithmic phase (6).

No pentoses were found to be active in the present survey nor in earlier work with the same tissue in natural media (7). This is in contrast to results obtained recently with a cell strain of malignant origin (5). Since it has been shown that the hexosemonophosphate shunt is of more importance in some tumors than in the corresponding normal tissues (14, 36), this observation may represent an important difference in cell types. Unfortunately, the positive results with ribose and ribose-5-phosphate in malignant strains were obtained in the presence of serum protein and the possible involvement of enzymes from the medium cannot be discounted.

The action of externally supplied enzymes can be a major complication in the interpretation of nutritional studies. For example, serum has been shown to contain maltase (7, 37), and the utilization of maltose by chick embryonic heart cultures has been assumed to be due entirely to the presence of this enzyme in the medium (7). In the present studies, where no external protein was supplied, and the cells were washed and depleted before use, maltose was still able to substitute for glucose to a considerable extent. It is apparent, therefore, that these cells do contain maltase.

Consideration of groups of related compounds, tested in this survey, has suggested metabolic steps which warrant further study. However, when the survey as a whole is examined, one striking pattern emerges. It can be seen, from Table I, that compounds on the Embden–Meyerhof pathway are progressively less efficient as glucose substitutes. Thus, a decreasing order of efficiency is obtained with glucose-6-phosphate, fructose-6-phosphate, and fructose diphosphate (the latter being completely negative). Also, a major defect in metabolism, in these cultures, appears to be established at the phosphofructokinase level under the present conditions. None of the compounds lying below this step were utilized, nor were any of the members of the citric acid cycle, although all these substances are implicated in normal glucose metabolism.

Acknowledgments

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References

1. Pomerat, C. M. and Willmer, E. N. J. Exptl. Biol. **16**, 222 (1939).
2. Wilson, H., Jackson, E. B., and Brues, A. M. J. Gen. Physiol. **25**, 689 (1941–42).
3. Astrup, T., Fischer, A., and Ohlenschlager, V. Acta Physiol. Scand. **13**, 267 (1947).
4. Fischer, A. Scientia, **83**, 170 (1948).
5. Eagle, H., Barban, S., Levy, M., and Schulze, H. O. J. Biol. Chem. **233**, 551 (1958).
6. Bailey, J. M., Gey, G. O., and Gey, M. K. J. Biol. Chem. **234**, 1042 (1959).
7. Harris, M. and Kutsky, P. B. J. Cellular Comp. Physiol. **42**, 449 (1953).
8. Chang, R. S. and Geyer, R. P. Proc. Soc. Exptl. Biol. Med. **96**, 336 (1957).
9. Moore, A. E. and Wroblewski, F. Proc. Soc. Exptl. Biol. Med. **98**, 782 (1958).
10. Leighton, J., Kline, I., and Belkin, M. Cancer Research, **17**, 359 (1957).
11. Perske, W. F., Parks, R. E., Jr., and Walker, D. L. Science, **125**, 1290 (1957).
12. Coriell, L. L., Tall, M. G., and Gaskill, H. Science, **128**, 198 (1958).
13. Boxer, G. E. and Shonk, C. E. J. Biol. Chem. **233**, 535 (1958).
14. Kizer, D. E. and McCoy, T. A. Cancer Research, **19**, 307 (1959).
15. Morgan, J. F., Morton, H. J., Campbell, M. E., and Guerin, L. F. J. Natl. Cancer Inst. **16**, 1405 (1956).
16. Morgan, J. F., Campbell, M. E., and Morton, H. J. J. Natl. Cancer Inst. **16**, 557 (1955).
17. Morton, H. J., Pasieka, A. E., and Morgan, J. F. J. Biophys. Biochem. Cytol. **2**, 589 (1956).
18. Hanks, J. H. and Wallace, R. E. Proc. Soc. Exptl. Biol. Med. **71**, 196 (1949).
19. Morgan, J. F., Morton, H. J., and Parker, R. C. Proc. Soc. Exptl. Biol. Med. **73**, 1 (1950).
20. Hers, H. G. Biochim. et Biophys. Acta, **22**, 202 (1956).
21. Adcock, L. H. and Gray, C. H. Biochem. J. **65**, 554 (1957).
22. Horecker, B. L. and Hiatt, H. H. New Engl. J. Med. **258**, 177 (1958).
23. Villavicencio, M. and Barron, E. S. G. Arch. Biochem. Biophys. **67**, 121 (1957).
24. Pasieka, A. E., Morton, H. J., and Morgan, J. F. Can. J. Biochem. and Physiol. **36**, 171 (1958).
25. Paul, J. and Pearson, E. S. Exptl. Cell Research, **12**, 212 (1957).
26. Lieberman, I. and Ove, P. J. Biol. Chem. **233**, 634 (1958).
27. Kipnis, D. M., Helmreich, E., and Cori, C. F. J. Biol. Chem. **234**, 165 (1959).
28. Potter, V. R. and Auerbach, V. H. Lab. Invest. **8**, 495 (1959).
29. Landau, B. R., Baird, H. A., and Zottu, S. J. Biol. Chem. **233**, 1257 (1958).
30. Fitch, W. M., Hill, R., and Chaikoff, I. L. J. Biol. Chem. **234**, 1048, (1959).
31. Freedland, R. A. and Harper, A. E. J. Biol. Chem. **234**, 1350 (1959).
32. Ginsburg, V. and Neufeld, E. F. Abstracts of papers, 132nd meeting, Am. Chem. Soc. 1957. p. 27C.
33. Kalckar, H. M. Advances in Enzymol. **20**, 111 (1958).
34. Morton, H. J. and Morgan, J. F. In preparation.
35. Isselbacher, K. J. Science, **126**, 652 (1957).
36. Kit, S. Cancer Research, **16**, 70 (1956).
37. Lieberman, I. and Eto, W. H. J. Biol. Chem. **225**, 899 (1957).

RESPIRATORY CARRIERS AND THE NATURE OF THE REDUCED DIPHOSPHOPYRIDINE NUCLEOTIDE OXIDASE SYSTEM IN XANTHOMONAS PHASEOLI[1]

R. M. Hochster and C. G. Nozzolillo

Abstract

Intact cells and cell-free extracts of the phytopathogenic organism *Xanthomonas phaseoli* have been shown to contain flavoprotein and the respiratory carriers: cytochrome b_1, cytochrome a_1, and cytochrome a_2. The reduced forms of these respiratory pigments are produced upon addition to a clear extract of substrate amounts of DPNH.

The highly active DPNH oxidase system in extracts of this organism has been studied as to requirements for inorganic ions, optimum pH, product formation, distribution, and solubilization. Carbon monoxide inhibits the terminal oxidation system; this effect is reversed by bright light.

An inhibitor study has shown members of the phenothiazine family of compounds to be most effective, followed by amytal, cyanide, BAL, atabrine, and *p*CMB. The most notable of the substances which did not inhibit were antimycin A, one of the quinoline-N-oxides, and azide.

The possibility exists that H_2O_2 may also be formed during the oxidation of DPNH although clear-cut evidence for its presence was difficult to obtain. *X. phaseoli* extracts do not contain a DPNH peroxidase. They exhibit, however, some DPNH – cytochrome *c* reductase activity which is believed to be quite independent of the DPNH oxidase system. The extracts are devoid of cytochrome *c* oxidase activity although they contain a respiratory system which readily oxidizes *p*-phenylenediamine.

Introduction

The view is held in this laboratory that a fundamental knowledge of the enzyme systems involved in energy transformations may lead to valuable data that will be useful in the ultimate understanding of the process of phytopathogenicity. It was established previously (1) that cell-free extracts of the phytopathogenic organism *Xanthomonas phaseoli* (causing common blight of beans) contain an unusually active reduced diphosphopyridine nucleotide oxidase system. Inasmuch as no information is available in the literature regarding the components or the mechanism of the electron transport systems in phytopathogenic organisms, a study was undertaken which was designed to provide evidence for the participation of specific respiratory carriers and to gain some insight into the nature of the highly active reduced diphosphopyridine nucleotide oxidase system. The present paper is a report of this work.

Materials and Methods

Materials

The following substances used in this investigation were commercial preparations: DPNH,* *p*CMB, crystalline horse heart cytochrome *c* (Sigma Chemical

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Contribution No. 490, from the Microbiology Research Institute, Research Branch, Canada Department of Agriculture, Central Experimental Farm, Ottawa, Ontario.

*The following abbreviations are used: DPN, diphosphopyridine nucleotide; DPNH, reduced form of diphosphopyridine nucleotide; tris, tris(hydroxymethyl)aminomethane; $MgCl_2$, magnesium chloride; NH_2OH, hydroxylamine; CO, carbon monoxide; BAL, 2,3-dimercaptopropanol; A.D., alcohol dehydrogenase; O.D., optical density; H_2O_2 hydrogen peroxide; NaCN, sodium cyanide; DBI, N-β-phenethylformamidinyliminourea.HCl; SN5949, 2-hydroxy-3-(2-methyloctyl)-1,4-naphthoquinone; $Na_2S_2O_4$, sodium hydrosulphite.

Co.); lysozyme, isonicotinic acid hydrazide, crystalline alcohol dehydrogenase (Nutritional Biochemicals Corp.); alumina powder-type A301 (Aluminum Co. of America); antimycin A (Wisconsin Alumini Research Foundation); BAL (Mann Research Laboratories); kinetin (California Corp. for Biochemical Research); desamino-DPN (Pabst Brewing Co.); versene (Bersworth Chemical Co.); and amytal (Eli Lilly and Co.).

We gratefully acknowledge receipt of the following substances with their donors listed in parentheses: 2-heptyl-4-hydroxyquinoline-N-oxide (Dr. J. W. Lightbown, National Institute for Medical Research, London), promazine.HCl (Dr. J. Seifter, Wyeth Institute for Medical Research, Philadelphia), atabrine (Dr. M. L. Tainter, Sterling–Winthrop Research Institute, Rensselaer, N.Y.), reserpine (Drs. C. W. Murphy and H. O. Dillenberg, Ciba Co. Ltd., Montreal), chloropromazine, methopromazine maleate, levomepromazine maleate, prochlorperazine dimaleate, trimeprazine tartrate (Dr. Guy Marier, Poulenc Ltd., Montreal), DBI (Dr. H. S. Sadow, U. S. Vitamin Corp., New York), and SN5949 (Dr. K. E. Hamlin, Jr., Abbott Laboratories, Chicago).

Methods

In most of the experiments mentioned in this paper the change in DPNH concentration was measured as the decrease in optical density at 340 mμ determined in a Beckman DU spectrophotometer. Oxygen uptake or output was measured by standard Warburg respirometry. Inhibition experiments with carbon monoxide and its reversal by light were done according to the method of Slater (2). Many of the experiments with inhibitors were carried out manometrically because some inhibitors caused the formation of turbid solutions which were unsuitable for spectrophotometry.

For anaerobic spectrophotometric experiments standard Beckman DU cuvettes were modified as follows: to the open end of the cuvettes were sealed glass heads having two side arms for tipping and a ground-glass joint with venting tube similar to the top portion of classical Thunberg tubes. The cell compartment of the spectrophotometer was modified so as to permit these glass units to fit and, in order to eliminate the stray light problem, a special black, box-like cover was placed above the cuvette compartment. It was necessary to treat each tube as follows: a mixture of 95% N_2 + 5% CO_2 was carefully bubbled through the material in the main compartment of the cuvette for 3 minutes, the top was then replaced, and the entire assembly evacuated at the water pump for 5 minutes. $N_2 + CO_2$ was then bubbled through again for 30 seconds followed by final evacuation at the water pump for 3 minutes. After this treatment no more dissolved O_2 was present and no disappearance of DPNH occurred in the presence of the enzyme. The above technique was found to be more effective than prolonged evacuation at the water pump (30 minutes) due to loss of activity experienced when the latter method was used.

The authors wish to express their gratitude to Dr. Britton Chance for his hospitality in making the facilities of his laboratory available to one of us (R. M. H.) and for giving so generously of his time. The data presented as

Fig. 1 were obtained by and under the guidance of Dr. Lucile Smith using the split-beam recording spectrophotometric method (3, 4), and those in Fig. 2 were obtained by Dr. Chance using his elegant double-beam spectrophotometer (5) with liquid air attachment.

Cells of *X. phaseoli* were grown, harvested, and tested for strain purity as described previously (1). Cell-free, sonic extracts were obtained as follows: 7.2 g freshly harvested cells were suspended in a mixture of 6.0 ml 0.1 *M* tris buffer (pH 7.5) and 8.4 ml distilled water and treated with sonic oscillation in a Raytheon 200-watt, 10-kc oscillator for 5 minutes at a power output of 1.0 ampere at 4 to 10° C. The suspension was then centrifuged at 10,500 r.p.m. ($13{,}300 \times g$) at 0 to 4° C for 10 minutes and only the uppermost layer was used for the experiments reported here. Departures from the above method of centrifugation will be specifically referred to in the text.

Hydrogen peroxide was determined by a sensitive analytical method (6) capable of accurately measuring H_2O_2 in the range of 1 to 3 μg/ml in the presence of the cell-free bacterial extract. The manometric method (7) using ethanol as acceptor in the presence of catalase and measuring O_2 uptake was also employed and this was further extended by colorimetric analysis for acetaldehyde with acid 2,4-dinitrophenylhydrazine followed by strong alkali. Absorption measurements were made at 420 mμ.

Experimental and Results

Difference Spectra of Whole Cells by Direct Spectrophotometry

The difference spectra of *X. phaseoli* cells both in the Soret and visible portions of the spectrum are shown in Fig. 1, curves 1 and 3. They indicate (8) the presence of flavoprotein (trough at 460 mμ), cytochrome b_1 (peaks at 429, 560 mμ), cytochrome a_1 (peaks at 440, 597 mμ), and cytochrome a_2 (peak at 630, trough at 655 mμ). The peak at 530 mμ represents the β-bands of the cytochromes while the apparent peak at 624 mμ cannot, at present, be assigned to any of the known cytochromes although it is conceivable that it may be related to the cytochrome a_2 peak. Curves 2 and 4 give the corresponding difference spectra where the contents of one of each of the matched set of cuvettes was treated with CO for 30 seconds. They give evidence in support of the ability of the cytochromes of *X. phaseoli* to form CO-hemochromogens. Under the above conditions no evidence for a cytochrome of the "*c*" type was obtained.

The fact that the peak at 560 mμ appeared to be skewed to the left suggested that this peak could be a composite of two or perhaps more cytochrome pigments. By means of the sensitive scanning technique of Chance (5) and in the presence of liquid air (9) this peak has been resolved into three components (Fig. 2). The peaks at 557 and 564 mμ are probably two types of cytochrome *b* while the hump at 549 mμ suggests a component of the cytochrome "*c*" type. It is known, however, that cooling of hemoproteins to the temperature of liquid air results in the sharpening, splitting, and intensification of some of the absorption bands (9). Since pure cytochromes b_1, a_1, and a_2 from bacterial

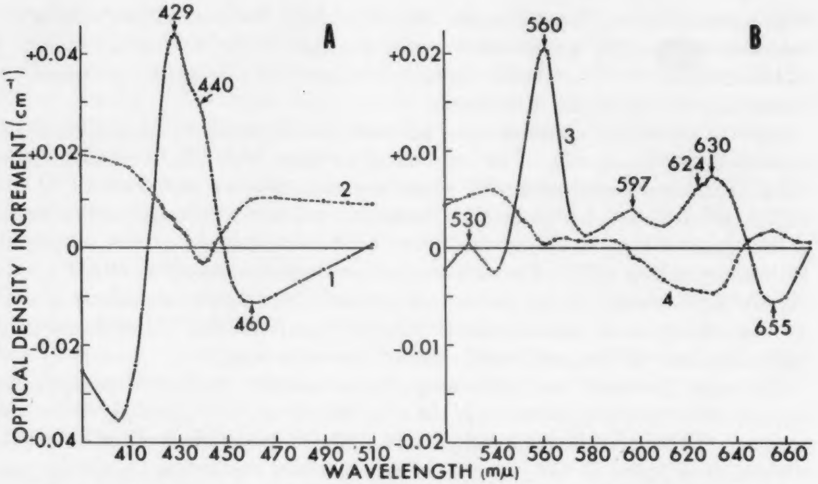

FIG. 1. Difference spectrum of *Xanthomonas phaseoli* (reduced-minus-oxidized, continuous lines, curves 1 and 3) and carbon monoxide difference spectrum (carbon monoxide compound-minus-reduced, dotted lines, curves 2 and 4).

The stock suspension of *X. phaseoli* cells (3 g solid-packed cells in 10 ml water) was diluted 25 times for measurements in the Soret region (A) and 10 times for measurements in the visible region of the spectrum (B).

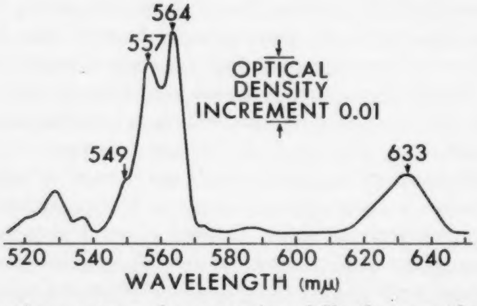

FIG. 2. Absorption spectrum of a suspension of *Xanthomonas phaseoli* at liquid air temperature.

The sample cuvette contained 1.0 ml of stock suspension of cells (1 g solid-packed cells in 5 ml water) which had been starved by aeration and gentle shaking for 1 hour prior to use, 1.25 ml glycerol, and a few crystals of $Na_2S_2O_4$. The reference cuvette contained the same but was without cells.

sources are not yet available for study, the interpretation of the above data must await the availability of these hemoproteins in pure form.

Difference Spectra of Cell-Free Extracts by Direct Spectrophotometry

Clear, cell-free extracts prepared by 15-minute sonic oscillation and subsequent centrifugation for 90 minutes at 40,000 r.p.m. (104,000×*g*) at 0° C were found to contain the same respiratory pigments as were shown in Fig. 1 for whole cells. Measurements were made in a Beckman DK-1 ratio recording spectrophotometer. When DPNH was added in substrate amounts (50 μmoles) to one cuvette, a tracing of the resulting reduced respiratory pigments was obtained which was identical with respect to the location of the respective peaks and troughs with flavoprotein and with cytochromes b_1, a_1, and a_2. This suggests that all the components of the respiratory chain found to be present in whole cells are probably involved in the DPNH oxidase system.

The Nature of the DPNH Oxidase System of Cell-Free Extracts

(a) Requirement for Inorganic Ions

Figure 3 shows the effects of several typical inorganic ions (A) and of versene (B) on the DPNH oxidase activity of extracts. Magnesium ions were always found to be most effective (Figs. 3A and 3B, curves 2) in bringing about maximal activity, while manganese was less useful and cobalt and nickel inhibitory at identical concentrations. The chelating agent versene was found to prevent magnesium activation (Fig. 3B, curves 1 and 4) unless excess magnesium was added (curve 5). The requirement for a divalent ion was thus established and magnesium ions were added to all subsequent experiments.

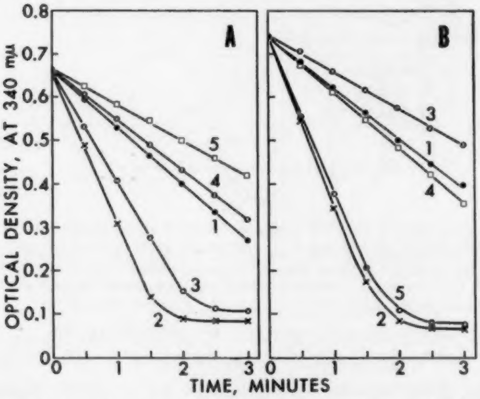

FIG. 3. Effects of inorganic ions and of versene on DPNH oxidase activity.

Cuvette contents in experiment A: tris buffer, pH 7.4, 50 μmoles; cell-free extract, 0.05 ml (2.25 mg protein); DPNH, 0.4 μmole; all inorganic ions, 10 μmoles. Total volume, 3 ml; temperature, 22° C. Curve 1 = DPNH control (no added ions), curve 2 = DPNH + Mg^{++}, curve 3 = DPNH + Mn^{++}, curve 4 = DPNH + Co^{++}, curve 5 = DPNH + Ni^{++}.

Cuvette contents in experiment B: phosphate buffer, pH 7.0, 50 μmoles; cell-free extract, 0.05 ml (2.25 mg protein); DPNH, 0.45 μmole; versene (where used), 10 μmoles. Total volume, 3 ml; temperature, 22° C. Curve 1 = DPNH control, curve 2 = DPNH + 10 μmoles Mg^{++}, curve 3 = DPNH + versene, curve 4 = DPNH + versene + 10 μmoles Mg^{++}, curve 5 = DPNH + versene + 50 μmoles Mg^{++}.

(b) Optimum pH

The DPNH oxidase of crude extracts was found to give maximal enzyme activity between pH 6.5 and pH 8.0, the absolute maximum usually being between pH 7.0 and pH 7.6 (Fig. 4).

(c) The Product of DPNH Oxidase Action

In the presence of 2 mg of enzyme protein, 0.4–0.5 μmole DPNH was reoxidized almost completely in 60 to 90 seconds. As shown in Fig. 5A, the addition of ethanol and of crystalline alcohol dehydrogenase brought the optical

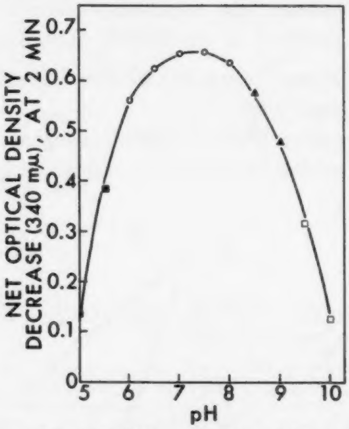

FIG. 4. Optimum pH of DPNH oxidase (crude) as determined by the spectrophotometric method, presented as the net O.D. decrease after 2 minutes of reaction.

Cuvette contents: buffers, 100 μmoles; $MgCl_2$, 10 μmoles; cell-free extract, 0.05 ml (2 mg protein); DPNH, 0.4 μmole. Total volume, 3 ml; temperature, 22° C.

Buffers used: acetate ■, phosphate ○, tris ▲, glycine–NaOH □.

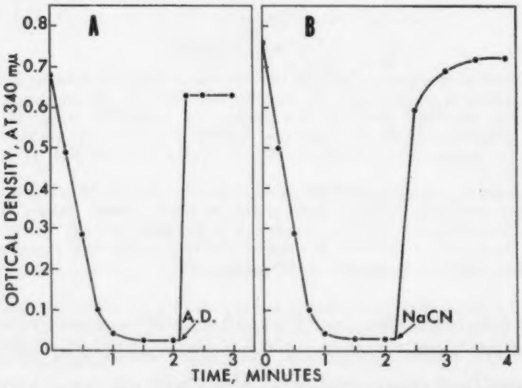

FIG. 5. DPN as the product of the DPNH oxidase reaction as shown by addition of alcohol plus alcohol dehydrogenase (A) and by formation of its cyanide complex (B).

Cuvette contents in experiment A: tris buffer, pH 8.4, 20 μmoles; $MgCl_2$, 10 μmoles; cell-free extract, 0.05 ml (2 mg protein); DPNH, 0.4 μmole. Total volume, 3 ml; temperature, 22° C. At 2 minutes (arrow, A.D.) the pH was adjusted to 9.2 with NaOH and immediately 20 μmoles ethanol and 1.0 mg of crystalline alcohol dehydrogenase were added to the sample and to the blank.

Cuvette contents in experiment B: phosphate buffer, pH 8.0, 20 μmoles; $MgCl_2$, 10 μmoles; cell-free extract, 0.05 ml (2 mg protein); DPNH, 0.45 μmole. Total volume, 3 ml; temperature, 22° C. At 2 minutes (arrow, NaCN) enough solid NaCN was added to raise the pH to 10.0. The ensuing values have been corrected for the spectral shift to 325 mμ caused by formation of the DPN–CN complex (10).

density nearly back to the initial value, proving that no significant degradation of DPN had occurred during the brief period of its formation. Further confirmation was obtained by treatment of another portion of the same sample with NaCN at pH 10.0 (Fig. 5B) to form the cyanide-addition complex (10) of DPN.

(d) Distribution of DPNH Oxidase

It was desirable to obtain the DPNH oxidase in a soluble form to facilitate eventually the purification of this enzyme system. Several techniques of cell breakage were tried. The results are compared in Table I. Lysozyme lysis of freshly harvested *X. phaseoli* cells in the presence of versene and tris buffer (pH 7.95) (11) resulted in a preparation in which 92% of the DPNH oxidase activity of the whole extract was contained in the insoluble particle fraction. The alumina-grinding method resulted in a preparation having considerably less activity (probably due to adsorption and to low cell breakage) and 63% of the recovered activity was found in the particulate fraction. Treatment in the sonic oscillator for 5 minutes gave a preparation in which 84% of the recovered DPNH oxidase activity was in the soluble supernatant fraction. Furthermore, it was found that solubilization increased with increasing time of oscillation.[*] If the DPNH oxidase activity is expressed as $\log\{a/(a-x)\}$, where a = the initial DPNH concentration and x = the concentration of DPNH converted to DPN, then a plot of this expression of activity against oscillation time results in a first-order kinetic relationship (Fig. 6). It is, therefore, quite reasonable to suggest that the DPNH oxidase system exists in the organism chiefly as an integral part of particulate matter which is brought into the soluble phase by means of the oscillation technique. Further studies of this phenomenon but with another enzyme system will appear in a subsequent paper from this laboratory.

TABLE I

Distribution of DPNH oxidase activity between various intracellular fractions obtained by three different methods of cell breakage

	Net µl O_2 uptake/15 min[*]		
Preparation	Lysozyme lysis (16 hr, at 0–2° C)	Alumina grinding (15 min, 0–4° C)	Sonic oscillation (10 kc, 5 min, 5° C)
Whole extract (13,000×*g*)	155	58	175
Whole extract spun at 104,000×*g*, resulting:			
(*a*) particles	143	17	27
(*b*) clear supernatant	13	10	138

NOTE: Vessel contents: phosphate buffer, pH 7.0, 100 µmoles; $MgCl_2$, 50 µmoles; original extract, 0.5 ml (or fraction diluted back); DPNH, 20 µmoles. Total volume, 3.0 ml; gas phase, air (KOH papers); temperature, 30° C.

[*] After separation, fractions were diluted back to the original volume.

(e) Carbon Monoxide and the Effect of Light

It was shown earlier (Fig. 1) that intact cells of *X. phaseoli* contain cytochromes b_1, a_1, and a_2 which were also shown to form CO-hemochromogens.

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[*] These extracts were prepared by Dr. N. B. Madsen of this Institute.

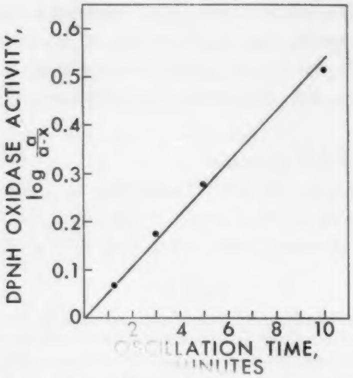

FIG. 6. First-order kinetics of the solubilization of DPNH oxidase as a result of sonic oscillation (a = initial DPNH concentration, x = concentration of DPNH converted to DPN).

Cuvette contents: phosphate buffer, pH 7.0, 50 μmoles; MgCl$_2$, 10 μmoles; DPNH, 0.4 μmole; supernatants from cell-free extracts (spun at 104,000×g for 90 minutes), 0.05 ml. Total volume, 3 ml; temperature, 22° C. Measurements made at 340 mμ and results calculated from experimental values after 2 minutes of reaction.

Since cytochromes a_1 and a_2 have been considered by some investigators (12, 13, 14, 15, 16) as the possible bacterial equivalents of mammalian cytochromes a and a_3 as the terminal oxidase, it was of interest to see whether CO would inhibit the DPNH oxidase of cell-free extracts.

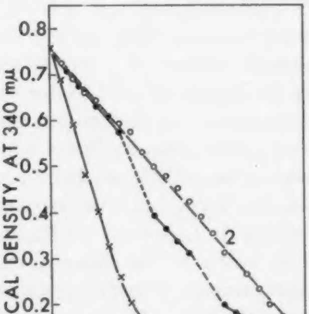

FIG. 7. Inhibition of DPNH oxidase activity by CO and its reversal by light.

Cuvette contents: phosphate buffer, pH 7.0, 70 μmoles; MgCl$_2$, 10 μmoles; cell-free extract, 0.02 ml (0.8 mg protein); DPNH, 0.45 μmole. Total volume, 3.0 ml; temperature, 23° C.

Curve 1 = DPNH oxidase control (in air); curve 2 = rate following treatment of mixture with CO in the dark for 2 minutes; curve 3 = effect on curve 2 of intermittent exposure to bright light (photoflood) for 1-minute periods, when extract was in the dark (shown by full lines) or in the light (dotted lines).

The results are shown in Fig. 7. In the experiment shown in curve 3 the cuvette contents were alternately kept in the dark (full lines) and exposed to the bright light (dotted lines) of a photoflood lamp for 1-minute periods. It is readily seen that when the mixture was held in the dark, DPNH oxidation proceeded at a rate similar to that of the inhibited reaction which was kept in the dark throughout (curve 2). When it was placed in the light the line thus obtained was very nearly parallel to the original, uninhibited reaction (curve 1). Bright light, therefore, effectively reverses the CO inhibition of the oxidase system.

TABLE II

Inhibitors of the DPNH oxidase system

Inhibitor	Concentration (in 3 ml reaction mixture)	pH of reaction mixture	Inhibition, % (calcd. after 15 minutes of reaction)
Amytal	5×10^{-4} *M*	7.65	35
	5×10^{-3} *M*	7.65	81
	5×10^{-2} *M*	7.65	100
Antimycin A*	4 μg†	7.4	0
	25 μg†	7.0	0
	100 μg	7.0	17
	100 μg	7.98	38
Atabrine	1×10^{-3} *M*	7.0	15
	5×10^{-3} *M*	7.0	55
2-Heptyl-4-hydroxy-quinoline-N-oxide	22 μg†	7.4	2
	45 μg†	7.4	6
	300 μg	7.98	15
	300 μg	6.75	10
Chloropromazine	1×10^{-4} *M*	6.75	38
	5×10^{-4} *M*	6.75	79
	1×10^{-3} *M*	6.75	94
	5×10^{-3} *M*	6.75	100
Promazine	1×10^{-3} *M*	6.75	68
	5×10^{-3} *M*	6.75	100
2,3-Dimercaptopropanol	2×10^{-3} *M*	7.0	39‡
	2×10^{-2} *M*	7.0	90‡
Isonicotinic acid hydrazide	5×10^{-3} *M*	7.0	0
p-Chloromercuribenzoate	1×10^{-3} *M*	7.98, 7.0	58
	5×10^{-3} *M*	6.75	47
Hydroxylamine	1×10^{-3} *M*	7.98, 7.0	0
	5×10^{-3} *M*	6.75	13
	5×10^{-2} *M*	7.0	17
Azide	1×10^{-3} *M*	7.98	18
	5×10^{-3} *M*	6.75	26
Kinetin	1×10^{-3} *M*	7.98	24
	5×10^{-3} *M*	6.75	18
Desamino-DPN	5×10^{-3} *M*	6.75	7
Reserpine*	Satd. soln.	7.0	0

NOTE: Vessel contents: phosphate buffer, 75 μmoles; $MgCl_2$, 50 μmoles; DPNH, 20 μmoles; sonic extract spun at 104,000×*g* for 90 minutes before use; supernatant, 0.3 ml (8 mg protein). Total volume, 3 ml; gas phase, air (KOH papers); temperature, 30° C.

*Added as a solution in 0.05 ml ethanol, ethanol correction made.

†Experiments carried out in the spectrophotometer.

‡Values corrected for the slight non-enzymatic reaction that occurred between DPNH and BAL.

(f) *Effect of Inhibitors*

Considerable experimental evidence is available on the effects of inhibitors on the DPNH oxidase of animal tissues but similar information on the corresponding bacterial enzyme system is sparse. Table II summarizes the results obtained with the 'soluble' DPNH oxidase of *X. phaseoli* extracts. Chloropromazine and promazine were found to be the most effective inhibitors. In fact, other members of the phenothiazine family of drugs (methopromazine maleate, levomepromazine maleate, prochlorperazine dimaleate, trimeprazine tartrate) were also most effective, although somewhat less than chloropromazine. Experiments with these substances were carried out at pH's below 7.0 in view of their relative insolubility above neutral pH. Amytal was found to be a good inhibitor at concentrations above 5×10^{-3} *M* and as stated previously (1) cyanide inhibited strongly but only at concentrations above 10^{-2} *M*. BAL also inhibited but required 2×10^{-2} *M* for 90% inhibition. It is thus quite clear that whatever the inhibitor used, high concentrations were necessary to achieve inhibitions of 90% or more.

Atabrine, *p*CMB, and kinetin were found to be less effective than the substances mentioned above. Little or no inhibition was obtained with hydroxylamine, azide, desamino-DPN, and isonicotinic acid hydrazide or with reserpine.

Antimycin A, long considered to be one of the most effective inhibitors of electron transport systems in animal tissues (18) where it is completely effective for the DPNH oxidase of heart muscle at concentrations of 0.14 μg/mg protein (19), was found to have no significant effect at all in our bacterial system at many times the concentration usually employed in animal tissue experiments. Some inhibition could be obtained, however, at a higher pH at approximately 100 times the usual concentration. Antimycin A was similarly ineffective with cell-free extracts of *E. coli* and of *Staphylococcus aureus* oxidizing succinate or cytochrome (20), and with digitonin extracts of rat liver mitochondria oxidizing DPNH (21). On the other hand, 2-heptyl-4-hydroxy-quinoline-N-oxide (20), a potent inhibitor of animal and of some bacterial tissue respirations, was equally ineffective at more than 100 times the concentration necessary to cause approximately 50% inhibition of the oxidation of succinate by extracts of *E. coli*. Negative results were also obtained with DBI and with a saturated solution of SN5949 in ethanol–water.

Inasmuch as antimycin A, BAL, SN5949, and the quinoline-N-oxides are believed to be involved in blocking the transfer of electrons in animal tissues in the same region (i.e. oxidation of cytochrome *b* by cytochrome *c*) (22) it is interesting to note that at the inhibitor concentrations employed here only BAL inhibited our bacterial system strongly. Thus, it is possible that, at least in the *X. phaseoli* system, BAL acts at a different site from the other three inhibitors mentioned above although a more detailed study is necessary to establish this point.

(g) *Stability of DPNH Oxidase*

The DPNH oxidase activity of extracts freshly prepared in the usual way from intact cells stored overnight at 0 to 4° C, or for several days at −20° C, is comparable to that of extracts prepared from newly harvested cells. Cells which have been lyophillized, however, yied extracts with an activity of only 10% of comparable fresh preparations. The activity of a number of extracts stored at 0 to 4° C was examined at intervals for up to 42 days and was found to be maintained at a high level during this period.

Catalase

As shown in Fig. 8A, sonic extracts of *X. phaseoli* contain an active catalase which was demonstrated under both anaerobic (curve 1) and aerobic conditions (curve 3) and which was readily inhibited by NH_2OH (curves 2, 4) at an inhibitor concentration (10^{-3} *M*) which had no effect at all on the DPNH oxidase system. This information is of considerable importance in the interpretation of the results given in a succeeding section of this paper.

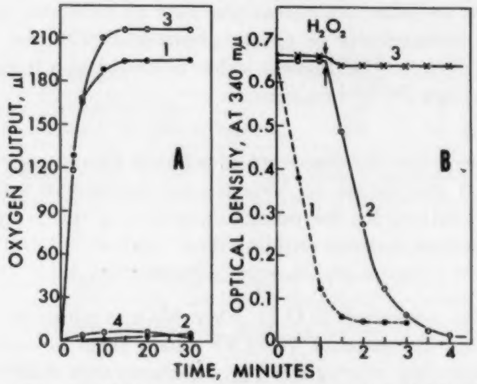

FIG. 8. Decomposition of H_2O_2 by *X. phaseoli* extracts and its inhibition by NH_2OH (A) and the effect of NH_2OH on the anaerobic disappearance of DPNH in the presence of H_2O_2 (B).

Vessel contents in experiment A: phosphate buffer, pH 7.0, 100 µmoles; $MgCl_2$, 50 µmoles; sonic extract, 0.3 ml (12 mg protein); H_2O_2, 19.2 µmoles; NH_2OH (where used), 3 µmoles. Total volume, 3 ml; temperature, 30° C. Curve 1 = catalase activity measured under anaerobic conditions (95% N_2 + 5% CO_2); curve 2 = as in curve 1 but with added NH_2OH; curve 3 = catalase activity measured aerobically (air, KOH papers); curve 4 = as in curve 3 but with added NH_2OH.

Cuvette contents in experiment B: phosphate buffer, pH 7.0, 100 µmoles; $MgCl_2$, 20 µmoles; sonic extract, 0.05 ml (2 mg protein); DPNH, 0.4 µmole; H_2O_2 (where used), 4.8 µmoles; NH_2OH (where used), 3 µmoles. Total volume, 3 ml; temperature, 23° C. Curve 1 = DPNH oxidation under aerobic conditions (to serve as control reaction rate); curve 2 = modified cuvette (see text) evacuated until no DPNH oxidation occurred (anaerobic); at arrow, H_2O_2 added from side arm; curve 3 = same as in curve 2, except that NH_2OH was present from the start.

Hydrogen Peroxide Formation

Incubation of 0.4 µmole DPNH at 30° C with 0.02 *M* phosphate buffer (pH 7.0), 0.003 *M* $MgCl_2$, with and without 0.001 *M* NH_2OH for periods of 0 to 80 seconds (stopped at 10-second intervals by the addition of molybdate in H_2SO_4 as required by the H_2O_2 test) under both aerobic and anaerobic conditions resulted in no net formation of H_2O_2 as measured by the iodide–molybdate method (6). These experiments were carried out with a variety of enzyme concentrations from 0.005 ml to 0.1 ml of extract and all gave completely negative results. The manometric method (7), which has recently been used also by others (23, 24) for the demonstration of H_2O_2 formation gave varied results. In some experiments the total O_2 uptake rose about 12% upon the addition of ethanol and could best be demonstrated under conditions where the concentration of extract used was low (approximately 0.5–1.0 mg protein) in order to ensure that the rate of O_2 uptake from DPNH was sufficiently slow. A further attempt to confirm the belief that small amounts of H_2O_2 may have been formed was made by chemical analysis for acetaldehyde in reaction mixtures used in the above catalase–EtOH manometric method. (It should be stated that at the enzyme concentrations used no evidence could be demonstrated either manometrically or spectrophotometrically, for the presence of alcohol dehydrogenase.) The highest value obtained was 0.6 μmole of acetaldehyde per 5.0 μmoles DPNH oxidized.

DPNH Peroxidase

In view of Dolin's (25, 26) discovery of a flavin-containing, hydroxylamine-insensitive DPNH peroxidase in *Streptococcus faecalis* it was of interest to examine our preparations for the possible presence of this enzyme.

Dolin's peroxidation reaction requires that

$$\text{DPNH} + \text{H}^+ + \text{H}_2\text{O}_2 \rightarrow \text{DPN}^+ + 2\text{H}_2\text{O}.$$

This should lead to a decrease in O.D. when H_2O_2 is added to DPNH anaerobically even in the presence of NH_2OH which does not act as a DPNH peroxidase inhibitor (25). Curve 3 of Fig. 8B shows that there was no decrease in O.D. under the conditions described. Thus there is no evidence for the presence or activity of a DPNH peroxidase in *X. phaseoli* extracts under the conditions employed.

The results shown in curves 1 and 2 of Fig. 8B are also of considerable interest. Curve 1 indicates the control rate of DPNH reoxidation under aerobic conditions. Curve 2 shows that anaerobiosis is complete (no drop in O.D. after evacuation) but that the addition of H_2O_2, in the absence of added NH_2OH, leads to a rapid drop in O.D. at a rate directly parallel to the control aerobic rate (curve 1). These results are explained as follows: In the absence of NH_2OH, the catalase of the extract remains uninhibited. Therefore, on addition of H_2O_2, catalase readily forms H_2O and $\frac{1}{2}O_2$ per mole of H_2O_2. The availability of free oxygen in this anaerobic system converts it to an aerobic one as seen not only by the fact that DPNH is now oxidized but also that the rate of oxidation exactly parallels the aerobic control. When NH_2OH is present (curve 3), however, catalase is completely inhibited; thus there is no oxygen available for these reactions to occur.

DPNH – Cytochrome c Reductase

It is well known that most bacterial oxidases do not oxidize reduced mammalian cytochrome *c* (27); in fact, the specificity of some bacterial cytochrome oxidases for their own bacterial cytochrome has been demonstrated in several laboratories (28, 29, 30). Exceptions have, however, been reported some examples of which are: *Micrococcus denitrificans* (31), *Escherichia coli* (32), and *Streptococcus faecalis* (25). Preparations from the latter organism were capable of completely reducing mammalian cytochrome *c*. Inasmuch as some slight evidence was obtained (Fig. 2) for the presence of a trace of a cytochrome of the "*c*" type (peak at 549 mμ as determined under liquid air conditions) in our organism, it was of interest to ascertain whether mammalian cytochrome *c* would be enzymatically reduced by DPNH since none of the organism's own cytochrome was available in purified form. The result is given in Table III.

TABLE III

Ability of DPNH to reduce mammalian cytochrome *c* in the presence of *X. phaseoli* extract

Expt. No.	System	Time elapsed, seconds	Net O.D.*	Enzymatic conversion to reduced cytochrome, %
1	Cytochrome *c*, extract, DPNH	300	0.105	28.4
2	Cytochrome *c*, extract, DPNH + $Na_2S_2O_4$	360	0.371	—

NOTE: Cuvette contents: tris buffer, pH 8.0, 50 μmoles; crystalline cytochrome *c*, 1.8 mg; sonic extract, 0.05 ml (2 mg protein). DPNH (0.4 μmole) added at zero time. Total volume, 3 ml; incubation temperature, 23° C. $Na_2S_2O_4$ added (experiment 2) at 300 seconds.

*Net increase in optical density at 550 mμ when DPNH was added to one of two otherwise identical cuvettes.

If it is assumed that the sodium hydrosulphite-treated system results in the complete reduction of all cytochrome *c*, then approximately 29% of the available cytochrome was reduced by the DPNH-linked enzyme system. Thus, some DPNH – cytochrome *c* reductase activity exists in *X. phaseoli* extracts.

Cytochrome c Oxidase

Reduced mammalian cytochrome *c* or ascorbate was not oxidized by cell-free extracts of *X. phaseoli*. It is of interest, however, that *p*-phenylenediamine was readily oxidized by extracts (Table IV), a reaction which remained unchanged by the addition of cytochrome *c*. It is realized that the lack of a catalysis by cytochrome does not rule out the participation of a terminal oxidase. On the contrary, it suggests that the system is probably saturated with respect to those natural bacterial electron carriers which function between *p*-phenylenediamine and oxygen. It remains for future experiments to establish their nature. The same rate of O_2 uptake with *p*-phenylenediamine as substrate was obtained also with a dialyzed preparation showing that the effect was due only

TABLE IV

Manometric demonstration of a terminal oxidase system in extracts of *X. phaseoli*

Additions	μl O_2/hr (blank subtracted)
None	0
p-Phenylenediamine	110
Ascorbate	0
Cytochrome *c*	5
p-Phenylenediamine + cytochrome *c*	111
Ascorbate + cytochrome *c*	1

NOTE: Vessel contents, *common system:* phosphate buffer, pH 7.0, 100 μmoles; versene, 2.7 μmoles; cell-free extract, 0.5 ml (20 mg protein); *additions:* cytochrome *c*, 0.25 μmole; ascorbate, 20 μmoles; *p*-phenylenediamine, 65 μmoles. Equivalent contents in the presence of boiled extract were used as controls. Gas phase, air (KOH papers); temperature, 30° C; total volume, 3 ml.

to *p*-phenylenediamine oxidation and not to other substrates which were undoubtedly present in our original cell-free extracts.

The *p*-phenylenediamine-catalyzed oxidation system may be a useful tool with which further progress may be made in the study of the terminal oxidases of phytopathogenic organisms.

Discussion

From the data presented in this paper, and taking into account evidence well documented in the literature (16, 27), it is most probable that the respiratory chain of *X. phaseoli* cells consists of

$$\text{flavoprotein} \rightarrow \text{cytochrome } b_1 \rightarrow \text{cytochrome } a_1 \rightarrow \text{cytochrome } a_2 \rightarrow O_2.$$

When DPNH is used as the substrate the results reported here indicate that, in addition to the above chain, alternate routes may exist which would have to be taken into account in an attempt to get a better assessment of the over-all picture. One of these is the possible formation of H_2O_2 *via* autoxidizable flavoproteins in cell-free extracts, a possibility which has already been referred to by Dolin (33). Any H_2O_2 thus produced would be immediately destroyed by the active catalase shown to be present and, therefore, would play no further role in DPNH oxidation. Our inability to demonstrate the presence of a DPNH peroxidase in *X. phaseoli* extracts supports this view. It is also possible that the DPNH – cytochrome *c* reductase activity described here represents a component of another possible alternate route.

Smith (27) has pointed out that the combination of cytochromes a_1 and a_2 is present in several bacteria and that "a_2 was never seen in the absence of a_1". This statement also applies to *X. phaesoli.* The spectrophotometric data (Fig. 1) suggest that the electron carrier system of *X. phaseoli* is similar to that in *Proteus vulgaris* and in *Azotobacter chroococcum* (27) as is its reaction with carbon monoxide.

Very little information exists in the literature regarding the requirement for inorganic ions by bacterial DPNH oxidases, with the exception of Dolin's (25) work on *Streptococcus faecalis.* In the latter case, Mn^{++} stimulated specifically while other divalent ions (e.g. Mg^{++}, Co^{++}, Zn^{++}, Ni^{++}, Fe^{++}) were totally without effect. In the *X. phaseoli* system a divalent ion is also required for maximum activity. Mg^{++} is the most effective (resulting in 3 times the initial reaction rate as compared with the control) while Mn^{++} is somewhat less active.

Solubilization of the initially particulate DPNH oxidase system of *X. phaseoli* was shown to be due to sonic oscillation. In a recent paper, Dolin (33) described work done with a 'soluble' DPNH oxidase from *Clostridium perfringens.* Since these cells were subjected to sonic treatment for 8 minutes, and only the uppermost layer used after centrifugation, it is very likely that the enzyme system studied by Dolin was also 'solubilized' by the oscillation technique as is shown in the present paper.

References

1. Hochster, R. M. and Katznelson, H. Can. J. Biochem. and Physiol. **36**, 669 (1958).
2. Slater, E. C. Biochem. J. **46**, 484 (1950).
3. Yang, C. C. and Legallais, V. Rev. Sci. Instr. **25**, 801 (1954).
4. Chance, B. Science, **121**, 621 (1955).
5. Chance, B. Rev. Sci. Instr. **22**, 619 (1951).
6. Savage, D. J. Analyst, **76**, 224 (1951).
7. Repaske, R. J. Bacteriol. **68**, 555 (1954).
8. Smith, L. Arch. Biochem. Biophys. **50**, 299 (1954).
9. Estabrook, R. W. J. Biol. Chem. **223**, 781 (1956).
10. Colowick, S. P., Kaplan, N. O., and Ciotti, M. M. J. Biol. Chem. **191**, 447 (1951).
11. Nozzolillo, C. G. and Hochster, R. M. Can. J. Microbiol. **5**, 471 (1959).
12. Negelein, E. and Gaerischer, W. Biochem. Z. **268**, 1 (1934).
13. Tissieres, A. Biochem. J. **50**, 279 (1951).
14. Tissieres, A. Nature, **169**, 880 (1952).
15. Chance, B. J. Biol. Chem. **202**, 383 (1953).
16. Castor, L. N. and Chance, B. J. Biol. Chem. **234**, 1587 (1959).
17. Ahmad, K., Schneider, H. G., and Strong, F. M. Arch. Biochem. **28**, 281 (1950).
18. Potter, V. R. and Reif, A. E. J. Biol. Chem. **194**, 287 (1952).
19. Slater, E. C. Biochim. et Biophys. Acta, **27**, 125 (1958).
20. Lightbown, J. W. and Jackson, F. L. Biochem. J. **63**, 130 (1956).
21. Lehninger, A. L., Wadkins, C. L., Cooper, C., Devlin, T. M., and Gamble, J. L., Jr. Science, **128**, 450 (1958).
22. Mahler, H. R. Ann. Rev. Biochem. **26**, 17 (1957).
23. Huennekens, F. M., Basford, R. C., and Gabrio, B. W. J. Biol. Chem. **213**, 951 (1955).
24. Cooper, J. A. D., Smith, W., Bacila, M., and Medina, H. J. Biol. Chem. **234**, 445 (1959).
25. Dolin, M. I. Arch. Biochem. Biophys. **55**, 415 (1955).
26. Dolin, M. I. J. Biol. Chem. **225**, 557 (1957).
27. Smith, L. Bacteriol. Rev. **18**, 106 (1954).
28. Kamen, M. D. and Vernon, L. P. Biochem. et Biophys. Acta, **17**, 10 (1955).
29. Tissieres, A. Biochem. J. **64**, 582 (1956).
30. Vernon, L. P. J. Biol. Chem. **222**, 1035 (1956).
31. Vernon, L. P. and White, F. G. Biochim. et Biophys. Acta, **25**, 321 (1957).
32. Brodie, A. F. J. Biol. Chem. **199**, 835 (1952).
33. Dolin, M. I. J. Bacteriol. **77**, 383 (1959).

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